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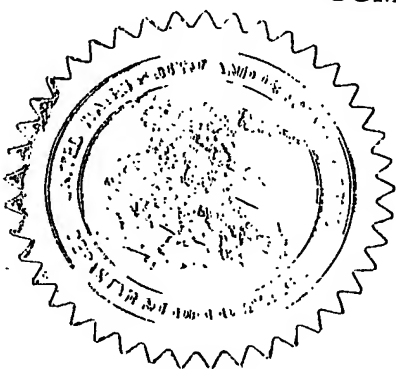
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This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).
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<input checked="" type="checkbox"/> Additional inventors are being named on the <u>1</u> separately numbered sheets attached hereto					
TITLE OF THE INVENTION (500 characters max)					
USE OF MYCOBACTERIAL VACCINES IN CD4 LYMPHOCYTE-DEFICIENT MAMMELS					
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ENCLOSED APPLICATION PARTS (check all that apply)					
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<input type="checkbox"/> Application Data Sheet. See 37 CFR 1.76					
METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT					
<input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27.					
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.					
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Respectfully submitted,

SIGNATURE

Date 01/23/2003

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REGISTRATION NO.
(if appropriate)
Docket Number:

44,704

96700/796

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Docket Number 96700/796		
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Number 1 of 1

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AR&E Docket No. 96700/796

New U.S. Provisional Patent Application (Large Entity Status)

Title: USE OF MYCOBACTERIAL VACCINES IN CD4 LYMPHOCYTE-
DEFICIENT MAMMALS

Inventors: William R. Jacobs, Jr.; Tsungda Hsu; Stoyan Bardarov;
Vasan Sambandamurthy; and Sheldon Morris

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USE OF MYCOBACTERIAL VACCINES IN CD4 LYMPHOCYTE-DEFICIENT
MAMMALS

Statement Regarding Federally Sponsored Research or Development

The U.S. Government has a paid-up license in this invention and the right in limited circumstances to require the patent owner to licence others on reasonable terms as provided by the terms of AI26170 awarded by National Institutes of Health.

Background of the Invention

(1) Field of the Invention

The present invention generally relates to live bacterial vaccines. More specifically, the invention is related to novel *Mycobacterium* sp. compositions, and the use of those compositions to protect mammals against disease caused by virulent *Mycobacterium* sp.

(2) Description of the Related Art

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- U.S. Patent No. 6,271,034.

U.S. Patent No. 5,504,005.

There exists an urgent need for a novel tuberculosis (TB) vaccine as there are more than 8 million new cases of tuberculosis and more than 2 million deaths reported each year by the WHO (Dye et al., 1999). The discovery of the causative agent of TB, *Mycobacterium tuberculosis*, by Robert Koch in 1882 opened up the possibility for a novel vaccine (Koch, 1882). Since then, numerous attempts to develop attenuated vaccines against tuberculosis have failed, including tuberculin (a protein extract of killed tubercle bacilli) developed by Dr. Koch himself. This failure of tuberculin to protect led to a "firm conviction that immunity could only be established by inducing a definite, albeit limited, tuberculosis process" Grange et al., 1983). Thus, numerous labs set out to follow the example of Dr. Louis Pasteur for viruses and enrich attenuated mutants of the tubercle bacillus following repeated passaging.

In order to test the hypothesis that a tubercle bacillus isolated from cattle (now known as *M. bovis*) could transmit pulmonary tuberculosis following oral administration, Drs. Calmette and Guerin developed a medium containing beef bile that enabled the preparation of fine homogenous bacillary suspensions (Calmette and Guerin, 1905). An *M. bovis* strain obtained from Dr. Norcard, was passaged every 21 days in this medium and after the 39th passage, the strain was found to be unable to kill experimental animal (Gheorghiu, 1996). "Between 1908 and 1921, the strain showed no reversion to virulence after 230 passages on bile potato medium" (Id.), which is consistent with the attenuating mutation being a deletion mutation. In the animal studies that followed, the strain ('BCG') was found to be attenuated but it also protected animals receiving a lethal challenge of virulent tubercle bacilli (Calmette and Guerin, 1920). BCG was first used as a vaccine against tuberculosis in 1921. From 1921 to 1927, BCG was shown to have protective efficacy against TB in a study on children (Weill-Halle and Turpin, 1925; Calmette and Plotz, 1929) and adopted by the League of Nations in 1928 for widespread use in the prevention of tuberculosis.

By the 1950's after a series of clinical trials, the WHO was encouraging widespread use of BCG vaccine throughout the world (Fine and Rodrigues, 1990). Although an estimated 3 billion doses have been used to vaccinate the human population against tuberculosis, the mechanism that causes BCG's attenuation remains unknown.

Mahairas et al. (1996) first compared the genomic sequences of BCG and *M. bovis* using subtractive hybridization and found that there were three major deletions (named RD1, RD2, and RD3) present in the genome of *M. bovis*, but missing in BCG. Behr et al. (1999) and others (Gordon et al., 2001) later identified 16 large deletions, including RD1 to RD3, present in the BCG genome but absent in *M. tuberculosis*. These authors concluded that 11 of these 16 deletions were unique to *M. bovis*, while the remaining 5 deletions were unique to BCG. They also found that one of these 5 deletions, designated RD1 (9454 bp), is present in all of the BCG substrains currently used as TB vaccines worldwide and concluded that the deletion of RD1 appeared to have occurred very early during the development of BCG, probably prior to 1921 (Behr et al., 1999).

The development of insertional mutagenesis systems for BCG and *M. tuberculosis* (Kalpana et al., 1991), transposon mutagenesis systems (Cirillo et al., 1991; McAdam et al., 1995; Bardarov et al., 1997) and allelic exchange systems (Balasubramanian et al., 1996; Pelicic et al., 1997) led to the isolation of the first auxotrophic (nutrient-requiring) mutants of these slow-growing mycobacteria. Auxotrophic mutants of BCG and *M. tuberculosis* have been shown to confer protection to *M. tuberculosis* challenges with variable efficacies (Guleria et al., 1996; Smith et al., 2001). However, a head-to-head comparison of BCG to a leucine auxotroph of BCG showed that a single immunization elicited no positive skin-test and imparted little immunity to challenges with *M. tuberculosis* or *M. bovis* (Chambers et al., 2000). In contrast, a methionine auxotroph of BCG that grows *in vivo* did confer significant protection to challenge to both *M. tuberculosis* and *M. bovis* (Id.). A single dose of a leucine

auxotroph of *M. tuberculosis* failed to elicit protection as good as BCG in BALB/c mice (Hondalus et al., 2000). These results suggest that optimal immunity against *M. tuberculosis* requires some growth of the immunizing strain. Double mutants of *M. tuberculosis* have also been created (Parish and Stoker, 2000), but whether such mutants are improved over single attenuating mutants in protecting mammals against challenge with a virulent mycobacterium, particularly when the host is immunocompromised, has not been established.

It is also worth noting that in the study of Chambers *et al.* (2000), both BCG and the BCG mutants seemed to protect better against *M. bovis* challenge than *M. tuberculosis*. If we assume the reverse correlate is true, we could hypothesize that optimal immunity against *M. tuberculosis* could be achieved with *M. tuberculosis*-derived mutant that grew in the mammalian host.

Based on the above, there remains a need for improved live mycobacterial vaccines having attenuated virulence, that confer protection from virulent mycobacteria, particularly *M. tuberculosis*. The need is particularly acute for immunocompromised individuals. The instant invention satisfies that need.

Summary of the Invention

The present invention is based on the discovery that mycobacteria having two attenuating mutations are safe and protect mammals that are deficient in CD4⁺ lymphocytes from challenge by virulent mycobacteria.

Thus, the present invention is directed to methods of treating a mammal that is deficient in CD4⁺ lymphocytes. The methods comprise inoculating the mammal with an attenuated mycobacterium in the *M. tuberculosis* complex. In these methods, the mycobacterium comprises two deletions, wherein a virulent mycobacterium in the *M. tuberculosis* complex having either deletion exhibits attenuated virulence.

Brief Description of the Drawings

FIG. 1 shows maps and autoradiographs pertaining to the construction of $\Delta RD1$ mutants of *M. tuberculosis*. Panel a, *M. tuberculosis* H37Rv published sequence between 4346 kb and 4364 kb, showing predicted *NcoI* sites. Arrows on the top represent the genes in the RD1 region. The RD1 region deleted from *M. bovis* BCG is represented by an open bar spanning from Rv3871 to Rv3879c. Upstream and downstream flanking sequences, UFS and DFS respectively, are indicated as closed bars underneath the grid line. Panel b, Southern hybridization of *M. tuberculosis* H37Rv $\Delta RD1$ created using two-step sequential homologous recombination. Panel c, Southern hybridization of *M. tuberculosis* H37Rv and Erdman $\Delta RD1$ strains created using specialized transduction.

FIG. 2 shows graphs summarizing experiments establishing that *M. tuberculosis* H37Rv $\Delta RD1$ is attenuated in SCID mice. Panel a, Seven female SCID mice were infected intravenously with 2×10^6 CFU *M. tuberculosis* H37Rv, *M. tuberculosis* H37Rv $\Delta RD1$, and *M. tuberculosis* H37Rv $\Delta RD1::2F9$ per mouse. The number of surviving mice was recorded post infection. Panel b, Mice were infected with different doses of *M. tuberculosis* H37Rv, *M. tuberculosis* H37Rv $\Delta RD1$, and *M. bovis* BCG. For each strain, infection doses of 2×10^6 CFU, 2×10^5 CFU, 2×10^4 CFU, and 2×10^3 CFU per mouse, were administered via tail intravenous injection.

FIG. 3 is photographs, micrographs and autoradiographs showing that the *M. tuberculosis* H37Rv $\Delta RD1$ mutant exhibits two distinct colonial morphotypes. Panel a, *M. tuberculosis* H37Rv. Panel b, *M. tuberculosis* H37Rv $\Delta RD1$. Panel c, *M. tuberculosis* H37Rv $\Delta RD1::2F9$. Panel d, Southern analysis of *M. tuberculosis* H37Rv $\Delta RD1$ *NcoI*-digested genomic DNA, isolated from three smooth and three rough colonies and probed with DFS. Panels e-g, Colonial morphotypes at higher magnification. e, Smooth morphotype at week 4. f, Rough morphotype at week 4. g, Rough morphotype at week 6.

FIG. 4 is graphs showing the growth kinetics of *M. tuberculosis* H37Rv $\Delta RD1$ in BALB/c mice. Mice were infected with 2×10^6 CFU through tail

injection. Time to death was noted and at day 1, week 4, 8, 14, and 22 post-infection, mice were sacrificed to determine the mycobacterial burden in the spleen, liver, and lung. The numbers represent the means of CFUs in organs derived from three animals. The error bars represent the standard errors of the means. Panel a, Time to death assay in BALB/c mice. Panel b, Spleen. Panel c, Liver. Panel d, Lung.

FIG. 5 is micrographs from pathological studies of infected BALB/c mice. Panels a – c, Lungs from mice infected with 2×10^6 CFU of *M. tuberculosis* H37Rv examined at 4, 8 and 14 weeks post-infection. The mild to moderate pneumonia at 4 and 8 weeks (a and b) progressed to severe consolidating granulomatous pneumonia at 14 weeks post infection (c). Panels d – f, Lungs from mice infected with 2×10^6 CFU of *M. tuberculosis* H37Rv $\Delta RD1$ examined at 4, 8 and 22 weeks post-infection showing moderate pneumonia at 8 weeks post-infection (e) and persistent bronchitis and multifocal pneumonitis at 22 weeks post-infection (f). Panels (g)– (i), Mild lung lesions from mice infected with 2×10^6 CFU of BCG at 4, 8 and 22 weeks post-infection. Mild focal granulomas scattered widely in the lung at each time point with predominately lymphocytic accumulations in foci at 22 weeks post-infection.

FIG. 6 shows graphs summarizing experiments establishing that pantothenate auxotrophy leads to attenuation of *M. tuberculosis* $\Delta panCD$ mutant in SCID mice. Panel A, Survival of BALB/c SCID mice ($n = 12$ per group) infected intravenously with 490 CFU of H37Rv (○) or 210 CFU of *panCD* complementing strain (*panCD* in single copy integrated into the chromosome)(●) or 3.4×10^5 CFU of $\Delta panCD$ mutant (▲) or 3.3×10^5 CFU of BCG-P (□). Panel B, Bacterial numbers in the spleen (●) and lungs (▲) of SCID mice infected intravenously with 490 CFU of H37Rv or numbers in spleen (○) and lungs (Δ) of mice infected with 3.4×10^5 CFU of $\Delta panCD$ mutant. The results represent means \pm standard errors of four to five mice per group.

FIG. 7 shows graphs summarizing experiments demonstrating the attenuation, limited growth and persistence of $\Delta panCD$ mutant in

immunocompetent mice. Panel A, Survival of BALB/c mice ($n=12$ per group) infected with 4.4×10^6 CFU of wild-type *M. tuberculosis* H37Rv (○), 3.2×10^6 CFU *panCD* complementing strain (*panCD* in single copy integrated into the chromosome)(●) or 2.4×10^6 CFU *panCD* mutant (▲). Panels B and C, Bacterial loads in spleen and lungs of BALB/c mice infected intravenously with 4.4×10^6 CFU wild-type H37Rv (○) or 3.2×10^6 CFU *panCD* complementing strain(●) or 2.4×10^6 CFU Δ *panCD* mutant (▲). CFUs were assayed at various time points on 7H11 agar with or without pantothenate supplementation where required. The results represent means \pm standard errors of four to five mice per group.

FIG. 8 shows graphs summarizing experiments demonstrating the attenuation, limited replication and persistence of Δ *nadBC* mutant in immunocompetent mice. Panels A and B, Bacterial loads in lungs and spleen of C57BL/6 mice infected with wild type *M. tuberculosis* H37Rv (●) or Δ *nadBC* mutant (○). Mice were infected intravenously with 10^6 CFU of each strain. CFUs were assayed at various time points on 7H11 agar with or without nicotinamide supplementation where required. The results represent means \pm standard errors of four to five mice per group. Panel C, Survival of C57BL/6 mice ($n=12$ per group) infected with 10^6 CFU of wild-type bacteria (●) or 10^6 CFU of Δ *nadBC* mutant (○).

FIG. 9 shows an illustration, map and autoradiograph relating to the pathway for the biosynthesis of pantothenic acid and coenzyme A and its disruption in *M. tuberculosis*. Panel a. The enzymes involved in the biosynthesis of pantothenic acid and having annotation in the genomic sequence of *M. tuberculosis* H37Rv are shown in bold numbers: 1) *panB*, ketopantoate hydroxymethyl transferase; 2) *panD*, aspartate-1-decarboxylase; 3) *panC*, pantothenate synthetase; 4) *panK*, pantothenate kinase; 5) *acpS*, ACP synthase. Panel b. Map of the *panCD* genomic region in the wild type *M. tuberculosis* H37Rv and the Δ *panCD* mutant. Restriction sites and probe location are indicated. Panel c. Southern blot of *Bss*HII-digested genomic DNA from

wild-type H37Rv (lane 1), two independent clones of $\Delta panCD$ mutant from H37Rv (lanes 2 & 3) and probed with a 716 bp downstream region flanking the *panCD* operon. Molecular size marker (in kb) is shown on the left.

FIG. 10 shows graphs summarizing experiments demonstrating that pantothenate auxotrophy leads to attenuation of $\Delta panCD$ mutant in mice. a. Survival of BALB/c SCID mice ($n = 12$ per group) infected intravenously with H37Rv (○) or *panCD*-complemented strain (●) or $\Delta panCD$ mutant (▲) or *M. bovis* BCG-P (□). b. Bacterial numbers in the spleen (○), liver (□) and lung (Δ) of SCID mice infected intravenously with H37Rv or the bacterial numbers in the spleen (●), liver (■) and lung (▲) of mice infected with $\Delta panCD$ mutant. c. Survival of immunocompetent BALB/c mice ($n = 16$ per group) infected with H37Rv (○) or *panCD*-complemented strain (●) or $\Delta panCD$ mutant (▲). d, e, f. Bacterial numbers in lung (d), spleen (e) and liver (f) of immunocompetent BALB/c mice infected intravenously with either H37Rv (○), *panCD*-complemented strain (●) or $\Delta panCD$ mutant (▲). Data are means \pm standard errors of four to five mice per group.

FIG. 11 shows micrographs and graphs summarizing experiments demonstrating that the $\Delta panCD$ mutant produces less tissue pathology in lungs of infected BALB/c mice and protects mice against challenge with virulent *M. tuberculosis*. a. Severe consolidating granulomatous pneumonia (★) obliterating the normal lung parenchyma at 3 weeks post-infection with H37Rv. b. Severe consolidating granulomatous pneumonia (★) obliterating the normal lung parenchyma at 3 weeks post-infection with the *panCD*-complemented strain, similar to the wild type strain. c. Mild lung infection caused by the $\Delta panCD$ mutant at 3 weeks post-infection. Localized multifocal granulomas (arrows) scattered widely in the lung. Most of the lung is normal alveolar spaces and airways. d. Lung of mouse infected with $\Delta panCD$ mutant examined histologically at 23 weeks post-infection. Occasional focal, mild perivascular and interstitial infiltrations composed of predominately lymphocytes (arrows). Most of the lung is normal alveolar spaces and airways. e, f. The attenuated

$\Delta panCD$ mutant protects mice against aerogenic challenge with virulent *M. tuberculosis* Erdman. Subcutaneously immunized mice were challenged after 90 days through the aerosol route. The CFU numbers reflect the bacterial burden at 28 days post aerosol challenge in the lung (e) and spleen (f). Naive mice - black fill; mice infected with 1 dose *panCD* - light shade; mice infected with 2 doses *panCD* - dark shade; mice infected with BCG-P - unshaded.

FIG. 12 shows autoradiographs of Southern analysis of the *NcoI*-digested genomic DNA isolated from the wild type and the $\Delta RD1$ mutants generated using specialized transduction in *M. tuberculosis* and *M. bovis*. Lanes: 1 - *M. tuberculosis* H37Rv; 2 - *M. tuberculosis* H37Rv $\Delta RD1$; 3 - *M. tuberculosis* Erdman; 4 - *M. tuberculosis* Erdman $\Delta RD1$; 5 - *M. tuberculosis* CDC1551; 6 - *M. tuberculosis* CDC1551 $\Delta RD1$; 7 - *M. bovis* Ravenel; and 8 - *M. bovis* Ravenel $\Delta RD1$. The probed used in the Southern analysis was either DFS (left) or IS6110-specific sequence (right).

FIG. 13 shows graphs summarizing data confirming that deletion of *RD1* in *M. tuberculosis* and *M. bovis* confers an attenuation of virulence for *M. tuberculosis* and *M. bovis*, as indicated by these Time to death curves of mice infected intravenously with 2×10^6 CFU mycobacteria. Panel A, SCID mice infected with *M. tuberculosis* H37Rv (■), *M. tuberculosis* H37Rv $\Delta RD1$ (□), *M. tuberculosis* Erdman (●), *M. tuberculosis* Erdman $\Delta RD1$ (○), *M. tuberculosis* CDC1551 (▲), *M. tuberculosis* CDC1551 $\Delta RD1$ (Δ), *M. bovis* Ravenel (▼), *M. bovis* Ravenel $\Delta RD1$ (∇); Panel B, SCID mice infected intravenously with *M. tuberculosis* H37Rv (●), *M. tuberculosis* $\Delta RD1$ (■), *M. tuberculosis* $\Delta RD1::2F9$ (▲), *M. bovis* Ravenel (○), *M. bovis* Ravenel $\Delta RD1$ (□), and *M. bovis* BCG (Δ); Panel C, BALB/c mice were infected with *M. tuberculosis* H37Rv (○), *M. tuberculosis* $\Delta RD1$ (Δ), and *M. bovis* BCG (□).

FIG. 14 are graphs summarizing experiments demonstrating the clearance of the lysine auxotroph in SCID mice. The viable bacterial counts are shown for the spleens, livers, and lungs of SCID mice injected intravenously with the lysine auxotroph strain and the prototrophic control strain. Three mice

were assayed at each time point. The error bars indicate the standard deviations of the mean values. Note that the counts at time zero are the counts obtained at 24 hours post-injection, as described in Example 5. Panels A, B and C show the log of the viable bacteria in each organ after injection with 1×10^7 CFU of the *Lys*⁻ *M. tuberculosis* mutant mc²3026 (□), or 1×10^7 CFU of the complemented *Lys*⁺ *M. tuberculosis* strain mc²3026/pYUB651 (■).

FIG. 15 is graphs summarizing experimental results of experiments that establish the vaccine efficacy of the *M. tuberculosis* lysine auxotroph mc²3026. C57Bl/6 mice were injected intravenously with 1×10^6 CFU of the *M. tuberculosis* lysine auxotroph mc²3026, followed by one or two additional injections at 4 week intervals. Five mice were sacrificed weekly after each immunization and the viable bacteria counts of the auxotroph determined in the lungs and spleens. Control mice were given a similar amount of BCG-Pasteur or only PBST. Shown in Panel A is the clearance of the auxotroph from the lungs of the mice after each immunization period; one injection (■), two injections (◆), and three injections (●). Three months after the initial immunization the vaccinated and control mice were challenged with virulent *M. tuberculosis* Erdman by the aerosol route. Five challenge mice were sacrificed following the challenge period and the lung homogenates plated to check the viable counts of the challenge inoculum. Groups of vaccinated and control mice were sacrificed at 14, 28, and 42 days later and the lung and spleen homogenates plated to determine viable colony forming units. Shown in Panel B are the viable challenge bacteria per lung of mice given one dose of the *M. tuberculosis* lysine auxotroph, and in panel C, the viable challenge bacteria per lung of mice given two doses of the auxotroph. Key: Viable challenge bacteria per lung of mice given the *M. tuberculosis* lysine auxotroph mc²3026 (■), BCG-Pasteur (◆), or PBST (○). P values are indicated in the figure. Note that the results shown here are for the lungs. Similar results (not shown) were obtained from the spleens in all the experiments.

FIG. 16 shows a graph summarizing experiments establishing the survival curves of mice immunized three times with the *M. tuberculosis* lysine auxotroph mc²3026. C57BL/6 mice were injected intravenously with 1×10^6 CFU of the *M. tuberculosis* lysine auxotroph mc²3026, followed by two more injections at 4 week intervals, and challenged as described in Example 5. The percent survival is shown above for mice immunized thrice with the *M. tuberculosis* lysine auxotroph mc²3026 (■, 5 mice total), once with BCG-Pasteur (◆, 5 mice), and for the PBST controls (●, 10 mice).

FIG. 17 shows graphs summarizing experimental results establishing that the virulence of strain mc²6030 is highly attenuated in SCID mice and BALB/c mice.

FIG. 18 shows graphs summarizing experimental results measuring growth of various strains of *M. tuberculosis* in spleen (Panel A) and lungs (Panel B) of C57BL/6 mice.

FIG. 19 is a graph summarizing experimental results establishing that immunization with mc²6020 and mc²6030 protects mice against TB as effectively as BCG. This graph shows the survival of C57BL/6 mice challenged with virulent *M. tuberculosis* Erdman through the aerosol route three months after a single dose subcutaneous immunization with either BCG, mc²6020 (Δ lysA Δ panCD) or mc²6030 (Δ RD1 Δ panCD) and compared to non-immunized naive mice. There were 12 to 15 mice in each survival group.

FIG. 20 is a graph summarizing experimental results establishing that immunization with mc²6030 protects CD4 deficient mice from TB. CD4^{-/-} mice were immunized by a single subcutaneous injection of 10^6 CFU of either Δ RD1 Δ panCD (mc²6030) or BCG-P and three months later challenged with 100-200 CFU of virulent *M. tuberculosis* through the aerosol route and compared to non-immunized (naive) CD4^{-/-} controls. Each group consists of 10 mice.

FIG. 21 shows graphs summarizing experimental results establishing that *M. tuberculosis* double deletion mutants are highly attenuated in SCID mice. A dose of 10^5 mc²6020 or mc²6030 were intravenously inoculated into SCID mice

(10 per group) and time to death assessments were performed. While the same dose of *M. tuberculosis* and BCG killed mice in 40 or 90 days, respectively, the mice infected with mc²6020 or mc²6030 survived over 400 or 250 days, respectively.

Detailed Description of the Invention

The present invention is based on the discovery that mycobacteria having two attenuating mutations are safe and protect mammals that are deficient in CD4⁺ lymphocytes from challenge by virulent mycobacteria. See Example 6.

Thus, the present invention is directed to methods of treating a mammal that is deficient in CD4⁺ lymphocytes. The methods comprise inoculating the mammal with an attenuated mycobacterium in the *M. tuberculosis* complex. In these methods, the mycobacterium comprises two deletions, wherein a virulent mycobacterium in the *M. tuberculosis* complex having either deletion exhibits attenuated virulence.

The protection afforded by those double mutants in mammals deficient in CD4⁺ lymphocytes is surprising because it was previously believed that CD4⁺ lymphocytes were crucial in establishing immunity against tuberculosis (Jones et al., 1993; Scanga et al., 2000). The instant discovery establishes that such mutants would be expected to be safe and effective in providing protection against tuberculosis in individuals with highly compromised immunity, such as individuals with HIV infection or those taking immunosuppressant drugs (e.g., transplant patients).

The mammal can be inoculated with the mycobacteria in the methods of the present invention by any of a number of ways known in the art. These include oral ingestion, gastric intubation, or broncho-nasal-ocular spraying. Other methods of administration include intravenous, intramuscular, intramammary, or, preferably, subcutaneous or intradermal injection. The immunization dosages required can be determined without undue experimentation. One or two dosages of avirulent mycobacteria at $1-2 \times 10^6$

colony forming units (CFU) have previously been used, but other dosages are contemplated within the scope of the invention. Multiple dosages can be used as needed to provide the desired level of protection from challenge.

It is well known in the art that in order to elicit an immune response with a live vaccine such as an avirulent mycobacteria, it is preferred that the vaccine organism can sustain an infection in the immunized host, to provide a sustained exposure of the host's immune system to the organism. Therefore, in various preferred embodiments, the mycobacteria used in the methods of the invention are capable of sustaining an infection in the host. The ability to sustain infection can be measured without undue experimentation by any of a number of ways described in the art. With the mycobacteria used in the methods of the present invention, a preferred way of measuring sustained infection is by determining whether viable mycobacteria of the inoculated strain will remain resident in an immunocompetent mouse (e.g., BALB/c or C57BL/6 strain) for more than four weeks. More preferably, the inoculated mycobacteria will remain resident in the mouse for at least ten weeks. In the most preferred embodiments, viable mycobacteria of the inoculated strain will remain resident in the mouse for at least 20 weeks.

Preferably, the attenuated mycobacteria used in the methods of the invention are capable of protecting a mammal from challenge by a virulent *M. tuberculosis* complex mycobacteria. This ability can be determined by any of a number of ways provided in the literature. A preferred method is aerogenically treating an immunocompetent mouse with the virulent mycobacteria, as described in Examples 1 and 2. Aerogenic challenge is preferred because that most closely mimics natural infection. The skilled artisan would understand that the ability of an avirulent mycobacterium to protect a mouse from challenge from a virulent mycobacterium is indicative of the ability of the avirulent mycobacterium to protect a human, including a human child, from tuberculosis infection. A more stringent test of an avirulent mycobacterium to prevent infection by a virulent challenge is to use an immunocompromised

mammal, e.g. a SCID mouse or a mouse deficient in CD4 or interferon γ production.

The scope of the present invention includes the use of mycobacteria in the *M. tuberculosis* complex that comprise two attenuating deletions, where at least one of the attenuating deletion was made using genetic engineering. As discussed above, examples of such deletions include deletions of an RD1 region, deletions of a region controlling production of a vitamin, and deletions of a region controlling production of an amino acid. These mycobacteria include any in the *M. tuberculosis* complex, including *M. africanum*, *M. bovis* including the BCG strain and the subspecies *caprae*, *M. canettii*, *M. microti*, *M. tuberculosis* and any other mycobacteria within the *M. tuberculosis* complex, now known or later discovered. Preferred species are *M. bovis*, including the BCG strain, and *M. tuberculosis*, since those species are the most important as causes of mammalian diseases, such as tuberculosis in humans and *M. bovis* infection in cows.

In some aspects of these embodiments, at least one of the two deletions is of the RD1 region (see Example 1). Strains with these deletions can be determined by any means in the art, preferably by molecular genetic means, for example by hybridization methods (e.g., Southern blot using a probe from the RD1 region) or by amplification methods (e.g., PCR using primers to amplify a portion of the RD1 region). An example of an *M. tuberculosis* RD1 region (from H37Rv) is provided herein as SEQ ID NO:1. The skilled artisan could identify analogous RD1 regions from other mycobacteria in the *M. tuberculosis* complex without undue experimentation. Those RD1 regions would be expected to have strong homology to SEQ ID NO:1, at least 80% homologous to SEQ ID NO:1. However, it is to be understood that virulent mycobacteria in the *M. tuberculosis* complex can be rendered avirulent by deletions in a portion of the RD1 region. Therefore, use of non-naturally occurring mycobacteria in the *M. tuberculosis* complex that have a partial deletion in the RD1 region are envisioned as within the scope of the invention, provided the deletion can cause a virulent *M. tuberculosis* to become avirulent. It is expected that such *M. tuberculosis* with

partial *RD1* deletions can still sustain an infection in a mammal and protect against challenge by a virulent *M. tuberculosis*.

In embodiments where at least one of the deletions is in a region controlling production of a vitamin, the deletion can be in any genetic element leading to loss of production of the vitamin, including structural genes for enzymes involved in the biosynthesis of the vitamin, and genetic control elements such as promoters, enhancers, etc.

Deletion of a region controlling production of any essential vitamin or their precursors is contemplated as within the scope of the invention. As used herein, an essential vitamin is defined by its normal usage, that is, a small molecular weight compound that is required as a cofactor for the efficient function of an essential enzyme or enzymes. Nonlimiting examples include vitamin A, thiamin (B1), riboflavin (B2), nicotinic acid (niacin)/ nicotinamide/ nicotinamide adenine dinucleotide (NAD)/ nicotinamide adenine dinucleotide phosphate (NADP/coenzyme II), pantothenate (pantothenic acid/B5), pyridoxine (B6), folic acid, B12, biotin, C, D, E and K. Preferred vitamin targets for deletion include nicotinamide and pantothenate (see Example 2). Methods for determining whether a mycobacterium has deletions leading to the loss of production of any of these vitamins are within the scope of the art.

Deletions leading to the loss of any of these vitamins would be expected to lead to attenuated virulence of an otherwise virulent mycobacterium in the *M. tuberculosis* complex. Any of those strains would also be expected to sustain an infection in a mammal.

Preferred vitamin targets are pantothenate and nicotinamide adenine dinucleotide (NAD)(see Example 2). A preferred pantothenate deletion is of structural genes in the pantothenate biosynthetic operon, most preferably the pan C and D genes, the combined mutation being $\Delta panCD$. An example of a deletion of those genes is the deletion of the sequence from *M. tuberculosis* H37Rv provided herein as SEQ ID NO:2. Similarly, a preferred NAD deletion is in the structural genes of the NAD biosynthetic operon, most

preferably the *nadB* and *nadC* genes, the combined mutation being $\Delta nadBC$. An example of a deletion in those genes is the deletion of the sequence from *M. tuberculosis* H37Rv provided herein as SEQ ID NO:3.

In other embodiments, at least one of the attenuating deletions is of a region controlling production of an amino acid. Preferred examples include deletions of a region controlling production of proline, tryptophan, leucine or lysine. When the amino acid is lysine, a preferred deletion is a $\Delta lysA$ deletion, e.g., SEQ ID NO:4.

The mycobacterium deletions can be made by serial in vitro passage of a virulent *M. tuberculosis* (as the well-known *M. bovis* BCG was made) and selection for the desired deletion. More preferably, however, the deletion is made by genetic engineering, since such genetic methods allow precise control of the deletion being made.

Various methods of making deletions in mycobacteria are known in the art. Nonlimiting examples include specialized transduction (see, e.g., U.S. Patent No. 6,271,034, Example 1 and Example 2), and sequential two-step recombination (see Example 1). The latter method can usefully employ a *sacB* selective marker (Example 1).

Since, in preferred embodiments of the invention, the mycobacteria exhibit attenuated virulence and can sustain an infection in a mammal, these mycobacteria can usefully further employ a foreign DNA stably integrated into the genome of the mycobacteria, such that the mycobacteria can express a gene product coded by the foreign DNA. See, e.g., U.S. Patent 5,504,005.

Thus, the present invention has wide applicability to the development of effective recombinant vaccines in mammals deficient in $CD4^+$ lymphocytes against bacterial, fungal, parasite or viral disease agents in which local immunity is important and might be a first line of defense. Non-limiting examples are recombinant vaccines for the control of bubonic plague caused by *Yersinia pestis*, of gonorrhea caused by *Neisseria gonorrhoea*, of syphilis caused by *Treponema pallidum*, and of venereal diseases or eye infections caused by

Chlamydia trachomatis. Species of *Streptococcus* from both group A and group B, such as those species that cause sore throat or heart disease, *Neisseria meningitidis*, *Mycoplasma pneumoniae*, *Haemophilus influenzae*, *Bordetella pertussis*, *Mycobacterium leprae*, *Streptococcus pneumoniae*, *Brucella abortus*, *Vibrio cholerae*, *Shigella* spp., *Legionella pneumophila*, *Borrelia burgdorferi*, *Rickettsia* spp., *Pseudomonas aeruginosa*, and pathogenic *E. coli* such as ETEC, EPEC, UTEC, EHEC, and EIEC strains are additional examples of microbes within the scope of this invention from which foreign genes could be obtained for insertion into mycobacteria of the invention. Recombinant anti-viral vaccines, such as those produced against influenza viruses, are also encompassed by this invention. Recombinant anti-viral vaccines can also be produced against viruses, including RNA viruses such as Picornaviridae, Caliciviridae, Togaviridae, Flaviviridae, Coronaviridae, Rhabdoviridae, Filoviridae, Paramyxoviridae, Orthomyxoviridae, Bunyaviridae, Arenaviridae, Reoviridae or Retroviridae; or DNA viruses such as Hepadnaviridae, Paroviridae, Papovaviridae, Adenoviridae, Herpesviridae or Poxviridae.

The use of these methods for administering recombinant vaccines to protect against infection by pathogenic fungi, protozoa or parasites are also contemplated by this invention.

The avirulent microbes used in the methods of the present invention are also contemplated for use to deliver and produce foreign genes that encode pharmacologically active products that might stimulate or suppress various physiological functions (i.e., growth rate, immune-stimulating cytokines, blood pressure, etc.) in CD4⁺-deficient mammals. In such microbes, the recombinant gene encodes said pharmacologically active products.

By immunogenic agent is meant an agent used to stimulate the immune system of an individual, so that one or more functions of the immune system are increased and directed towards the immunogenic agent. Immunogenic agents include vaccines.

An antigen or immunogen is intended to mean a molecule containing one or more epitopes that can stimulate a host immune system to make a secretory, humoral and/or cellular immune response specific to that antigen.

In preferred embodiments, the foreign DNA encodes an antigen, an enzyme, a lymphokine, an immunopotentiator, or a reporter molecule. Preferred examples include antigens from *Mycobacterium leprae*, *Mycobacterium tuberculosis*, malaria sporozoites, malaria merozoites, diphtheria toxoid, tetanus toxoids, *Leishmania spp.*, *Salmonella spp.*, *Mycobacterium africanum*, *Mycobacterium intracellulare*, *Mycobacterium avium*, *Treponema spp.*, Pertussis, Herpes virus, Measles virus, Mumps virus, *Shigella spp.*, *Neisseria spp.*, *Borrelia spp.*, rabies, polio virus, human immunodeficiency virus, snake venom, insect venom, and *Vibrio cholera*; steroid enzymes; interleukins 1 through 7; tumor necrosis factor α and β ; interferon α , β , and γ ; and reporter molecules luciferase, β -galactosidase, β -glucuronidase and catechol dehydrogenase.

Preferred embodiments of the invention are described in the following examples. Other embodiments within the scope of the claims herein will be apparent to one skilled in the art from consideration of the specification or practice of the invention as disclosed herein. It is intended that the specification, together with the examples, be considered exemplary only, with the scope and spirit of the invention being indicated by the claims which follow the examples.

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Maniatis, Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual" (1989); "Current Protocols in Molecular Biology" Volumes I-IV (Ausubel, R. M., ed. (1997); and "Cell Biology: A Laboratory Handbook" Volumes I-III (J. E. Celis, ed. (1994).

Example 1. *Mycobacterium tuberculosis* having an *RD1* deletion has attenuated virulence and protects against tuberculosis

This example describes experimental methods and results that establish that deleting the *RD1* region from a virulent *M. tuberculosis* attenuates the virulence of the *M. tuberculosis* in both immunocompetent and immunocompromised mice, and protects against subsequent challenge by a virulent *M. tuberculosis*.

Materials and Methods

Media and Cultures. The mycobacterial strains *M. tuberculosis* H37Rv, *M. tuberculosis* Erdman and *M. bovis* BCG Pasteur were obtained from the Trudeau Culture Collection (Saranac Lake, NY). They were cultured in Middlebrook 7H9 broth and 7H10 agar supplemented with 10% OADC, 0.5% glycerol, and 0.05% Tween 80. Cyclohexamide, which does not affect mycobacterial growth, was added to the 7H10 agar medium at 0.1% to avoid fungal contamination. To examine the colony morphology of mycobacteria, Tween 80 was not added to 7H10 agar medium. The acriflavin resistant strain (Hepper and Collins, 1984) of *M. tuberculosis* Erdman grew in the presence of 20 µg of acriflavin per ml of medium.

DNA manipulation and construction of *M. tuberculosis* $\Delta RD1$. The following four primers were used to amplify upstream and downstream flanking sequences (UFS and DFS, respectively) for the construction of the *RD1* deletion mutants. UFS was amplified using TH201: GGGGGCGCACCTCAAACC and TH202: ATGTGCCAATCGTCGACCAGAA. DFS was amplified using TH203: CACCCAGCCGCCCGGAT, and TH204: TTCCTGATGCCGCCGTCTGA. Recognition sequences for different restriction enzymes were included at the ends of each primer to enable easier manipulation.

The unmarked deletion mutant of *M. tuberculosis* H37Rv, mc²4004, was generated by transformation (Snapper et al., 1988) using a *sacB* counterselection (Pelocic et al., 1996; Pavelka and Jacobs, 1999). Specifically, the plasmid pJH508 was created by first cloning UFS into *KpnI* and *XbaI* sites,

then cloning DFS into *EcoRI* and *HindIII* sites of pJH12, a pMV261-derived *E. coli*-Mycobacteria shuttle plasmid, to create pJH506 in which UFS and DFS flanked a green fluorescent protein gene (GFPuv, Clontech) whose expression was driven by the *M. leprae* promoter 18Kd. The UFS-gfp-DFS cassette was sub-cloned into the *EcoRV* site of plasmid pYUB657 to create pJH508. The first homologous recombination involved the identification of hygromycin resistant colonies, resulting from the transformation of *M. tuberculosis* with pJH508. Southern analysis of the *NcoI* digested DNA isolated from hygromycin resistant colonies probed with UFS or DFS, confirmed the presence of a single copy of pJH508 inserted into the *M. tuberculosis* genome. The transformant identified was then grown in 7H9 broth to saturation, to allow the second homologous recombination to occur, resulting in recombinants that could be selected by plating the culture on 7H10 plates, supplemented with 3% sucrose. Both Southern analysis and PCR of the DNA isolated from sucrose resistant colonies confirmed the RD1 deletion.

Specialized transduction (Bardarov and Jacobs, 1999), a mycobacteriophage-based method for the delivery of homologous DNA constructs using conditionally replicating shuttle phasmids (Jacobs et al, 1987; Bardarov and Jacobs, 1999; Carriere et al., 1997), has been used successfully for *M. tuberculosis* (Glickman et al., 2000; Glickman et al., 2001; Raman et al., 2001). Specifically, a transducing phage phAEKO1 was constructed by inserting UFS and DFS into pJSC347, flanking a hygromycin cassette, to create pJH313. pJH313 was digested with *PacI* and ligated to phAE159, a temperature sensitive mycobacteriophage derived from TM4. The transduction was performed by growing *M. tuberculosis* to an O.D.₆₀₀ of 0.8, washing twice with MP buffer, re-suspending into an equal volume of MP buffer and mixing with the transducing phage phAEKO1 at an MOI of 10. The mixtures were incubated at 37°C overnight, then plated on 7H10 plates supplemented with hygromycin at 50 µg/ml. Hygromycin resistant colonies were analyzed by PCR and Southern hybridization, as described above, to confirm the deletion of RD1.

Complementation analyses was performed using the integration proficient cosmids (Pascopella et al., 1994; Lee et al., 1991) pYUB412 made by S. Bardarov, a library made by F. Bange, and cosmid identified and generously provided by S.T. Cole.

Results

Genetic engineering of *M. tuberculosis* mutants with *RD1* deletions. The *RD1* (region of difference) region has been defined as the specific 9454 bp of DNA that is present in virulent *M. tuberculosis* and *M. bovis*, but absent in BCG (Mahairas et al., 1996). The annotation of *RD1* predicts that the deletion would disrupt 9 genes encoding ORF's (Id.; Cole et al., 1998). Five of the 9 ORF's have no known functions (Rv3871, Rv3876, Rv3877, Rv3878 and Rv3879c), two genes encode members of the PE/PPE family (Rv3872/Rv3873), and two genes encode the secreted proteins Cfp10 (Berhet et al., 1998) and Esat6 (Andersen et al., 1991)(Rv3875) (Fig. 1). To test if the *RD1* region is essential for virulence in *M. tuberculosis*, it was necessary to 1) delete the *RD1* region from virulent *M. tuberculosis* strains, 2) demonstrate loss of virulence and 3) restore virulence by complementation with the *RD1* DNA. The *RD1* deletion ($\Delta RD1$) was successfully introduced into *M. tuberculosis* by two different techniques, utilizing both a plasmid that allows two-step sequential recombination to make an unmarked deletion, and specialized transduction (Fig. 1a-c). For both methods, the same 1200 bp on each side of the *RD1* deletion were cloned into the appropriate plasmid or phage vector and then introduced into *M. tuberculosis* H37Rv by transformation or phage infection. An unmarked *RD1* deletion mutant of *M. tuberculosis* H37Rv, mc²4004, was constructed, purified, and has the advantage that additional mutations can be readily added to it. In addition, the *RD1* deletion was successfully engineered in the H37Rv and Erdman strains of *M. tuberculosis* using a specialized transducing phage. Since TM4 phages have been shown to infect over 500 clinical *M. tuberculosis* isolates (Jacobs et al., 1987), it should be possible to introduce the *RD1* deletion into any *M. tuberculosis* isolate of interest.

M. tuberculosis H37Rv $\Delta RD1$ is attenuated for virulence. To test if the *RD1* deletion causes an attenuating phenotype in *M. tuberculosis*, the *M. tuberculosis* H37Rv $\Delta RD1$ (mc²4004) was introduced into immunocompromised mice possessing the SCID (severe combined immunodeficiency) mutation. Groups of ten mice were injected intravenously with either 2×10^6 *M. tuberculosis* H37Rv or *M. tuberculosis* H37Rv $\Delta RD1$ and three mice per group were sacrificed 24 hours later to verify the inoculation doses. All of the SCID mice infected with the parental *M. tuberculosis* H37Rv strain died within 14 to 17 days post infection (Fig. 2a). In contrast, the SCID mice infected with the same dose of *M. tuberculosis* H37Rv $\Delta RD1$ were all alive at 35 days post-infection demonstrating a marked attenuation of the strain. To prove that the attenuation was due to the *RD1* deletion, mc²4004 was transformed with an integrating plasmid containing the *RD1* region from *M. tuberculosis* H37Rv. SCID mice injected intravenously with 2×10^6 of the transformed strain died 13 to 16 days post-infection (Fig. 2a), thereby, establishing that the genes in the *RD1* deletion complemented the attenuating phenotype.

To further characterize the attenuating phenotype of the *RD1* deletion in mc²4004, we compared the virulence of *M. tuberculosis* H37Rv and BCG-Pasteur to *M. tuberculosis* H37Rv $\Delta RD1$ with time-to-death experiments in SCID mice following injections with 10-fold varying inocula. Groups of 10 mice were injected intravenously, each mouse receiving from 2×10^3 to 2×10^6 CFU. Fig. 2b shows that the SCID mice succumbed to the infection with all three mycobacterial strains. However, the SCID mice succumbed to an *M. tuberculosis* H37Rv intravenous infection within 2 to 5 weeks, in a dose dependent manner. In the same time frame, the SCID mice did not succumb to infection with *M. tuberculosis* H37Rv $\Delta RD1$ until week 7, and only then, with the high dose of 2×10^6 CFU. Mice receiving 2×10^3 CFU *M. tuberculosis* H37Rv $\Delta RD1$ survived longer than 14 weeks post infection, the survival rate of which coincided with the mice receiving 2×10^6 CFU of *M. bovis* BCG. Thus, these experiments established that *M. tuberculosis* H37Rv $\Delta RD1$ was significantly more attenuated

than its parent, but not as attenuated as BCG-Pasteur in the immunocompromised mice.

Colonial morphotypes of *M. tuberculosis* H37Rv $\Delta RD1$. The *M. tuberculosis* H37Rv $\Delta RD1$ mutant was generated independently three times from the single crossover construct (mc²4000) and upon subculturing, consistently yielded a 20 to 50% mixture of two colonial morphotypes on Middlebrook medium without Tween 80 (Fig. 3a). One morphotype was a smooth (S) phenotype that was flat and corded (like the parental *M. tuberculosis* H37Rv strain) and the second was a rough and raised (R) phenotype. Repeated subculturing of either the R or S colonies continued to yield both colonial morphotypes, but with a distribution of approximately 80% smooth and 20% rough colonies. The distinction of these two types of morphology could be noted even when the colonies were less than two weeks old as the rough colonies were constricted and elevated with only a small portion of the base of the colony attached to the agar, while the smooth colonies tends to be flattened and spread out. When colonies grew older, e.g. 6 weeks old, the rough colonies remained more constricted compared to those of smooth colonies. The rough colonies exhibited large folds on the surface (Fig. 3f, g), as compared to those of the smooth colonies that exhibited small wrinkles (Fig. 3e).

Interestingly, in 1929, Petroff *et al.* reported a similar property for an early-derived BCG strain (Petroff *et al.*, 1929) and proposed that the attenuation phenotype of BCG was not stable. Calmette disputed that the avirulent phenotype reverted and postulated that Petroff *et al.* had acquired a contaminating virulent strain. Southern analysis of R and S colonies revealed each morphotype has the same *RD1*-deleted genotype (Fig. 3d). Furthermore, complementation of *M. tuberculosis* H37Rv $\Delta RD1$ with the *RD1* region restored the mutant phenotype back to the homogenous parental S phenotype (Fig. 3a-c). These results suggest that the variable morphotypes resulted directly from the *RD1* deletion thus dissociating a direct correlation of virulence with morphotype.

The *M. tuberculosis* H37Rv $\Delta RD1$ is highly attenuated in immunocompetent BALB/c mice. To further assess the pathogenicity, survival, growth kinetics, and the histopathological analysis of the *M. tuberculosis* H37Rv $\Delta RD1$ mutant, we compared the parental *M. tuberculosis* H37Rv to BCG-Pasteur strains in BALB/c mice. In survival studies, greater than 50% BALB/c mice had died at 14 weeks post i.v. infection with 2×10^6 CFUs of *M. tuberculosis* H37Rv strain (Fig. 4a). In contrast, all mice infected with a similar dose of either BCG or *M. tuberculosis* H37Rv $\Delta RD1$ survived for longer than 22 weeks. These results were substantiated in a separate experiment in which a group of 11 BALB/c mice were infected with 1×10^5 CFU of *M. tuberculosis* H37Rv $\Delta RD1$ and 9 of 11 mice (81%) survived greater than 9 months post-infection (data not shown). While BCG and *M. tuberculosis* H37Rv $\Delta RD1$ showed similar survival results, the growth relative kinetics in mouse organs differed substantially. BCG grew in a limited fashion in lungs, liver and spleen in BALB/c mice and was cleared to undetectable levels by week 12 (Fig. 4b-d). In contrast, the *M. tuberculosis* H37Rv $\Delta RD1$ strain grew in a fashion indistinguishable from the parental *M. tuberculosis* H37Rv in all mouse organs for the first 8 weeks. Thereafter, mice infected with the parental *M. tuberculosis* failed to contain the infection leading to mortality. Strikingly, mice infected with the *M. tuberculosis* H37Rv $\Delta RD1$ showed a definite control over infection resulting in significantly prolonged survival of mice (Fig. 4b-d).

The differing survival data of the three strains was clearly substantiated by histopathological analysis. *M. tuberculosis* H37Rv $\Delta RD1$ caused less severe organ damage in the lung, liver and spleen than the highly virulent parent strain *M. tuberculosis* H37Rv. *M. bovis* BCG was the least virulent of the three strains. Based on histopathological evaluation, the mortality in mice infected with the wild type *M. tuberculosis* H37Rv (documented above and in Fig. 4a) was caused by worsening pneumonia, hepatitis and splenitis (Fig. 5a-c). Mice examined at 14 weeks post-infection had developed severe lobar granulomatous pneumonia. Acid fast staining demonstrated large numbers of *M. tuberculosis* H37Rv, often

in clumps, throughout the lung. The livers and spleens showed a severe diffuse granulomatous inflammation.

Histopathological examination further demonstrated that *M. tuberculosis* H37Rv $\Delta RD1$ was attenuated in virulence compared to the parent strain *M. tuberculosis* H37Rv (Fig. 5d-f). In contrast to the rapidly progressive infection with the parent strain *M. tuberculosis* H37Rv, the lung lesions caused by *M. tuberculosis* H37Rv $\Delta RD1$ were maximal in mice examined at 8 weeks post-infection. Consolidating granulomatous pneumonia involved an estimated 25-30% of the lung in these mice. Numerous organisms were demonstrated by acid fast staining. The pneumonia subsequently underwent partial resolution. By 14 weeks, and again at 22 weeks post-infection, the lungs showed peribronchial and perivascular inflammatory cell accumulations and focal, generally non-confluent, granulomas now with a prominent lymphocytic infiltration. The numbers of acid fast organisms were reduced. Liver lesions consisted of low numbers of scattered granulomas. Spleens were smaller, with persistent granulomas in the red pulp.

Mice infected with *M. bovis* BCG showed mild lesions in the lung, liver and spleen at all time points (Fig. 5g-i). At longer time intervals post-infection the lesions were fewer in number, smaller with prominent lymphocytic infiltrations. At 14 weeks post-infection, *M. bovis* BCG was below the level of detection by acid fast staining. In summary, whereas *M. tuberculosis* H37Rv $\Delta RD1$ initially grew in a manner similar to the parental *M. tuberculosis* H37Rv, this $\Delta RD1$ mutant was limited in the extent of spread of infection, particularly in the lung. This contrasted to the extensive and severe damage caused by the parent strain. The subsequent resolving granulomas, localization of the lesions and changes in the composition of the inflammatory cell infiltrations suggested that the mice mounted an effective immune response to combat *M. tuberculosis* H37Rv $\Delta RD1$ infection and thereby reduced the numbers of viable organisms. *M. tuberculosis* H37Rv $\Delta RD1$ protects mice against aerosolized *M. tuberculosis* challenge. To test the potential of *M. tuberculosis* H37Rv $\Delta RD1$ to immunize

mice and protect against tuberculous challenge, we used the model of subcutaneous immunization followed by aerosol challenge with virulent *M. tuberculosis*. Our initial studies in C57BL/6 mice monitored the growth the *M. tuberculosis* H37Rv $\Delta RD1$ strain over an 84-day period. Groups of mice (5 mice per group) were vaccinated subcutaneously (sc) either once or twice, 6 weeks apart, with 10^6 CFU *M. tuberculosis* H37Rv $\Delta RD1$ organisms. Additional mice were infected intravenously (iv) with the same dose of the RD1-deleted strain in order to examine the pathogenicity in C57BL/6 mice.

As seen in Table 1, *M. tuberculosis* H37Rv $\Delta RD1$ persisted in the lungs, liver, and spleen for 3 months at moderate levels of infection but the organisms failed to grow substantially in the lungs and spleens of mice that had been inoculated iv. In contrast, reduced persistence and decreased concentrations of *M. tuberculosis* H37Rv $\Delta RD1$ organisms were detected in organ homogenates prepared from mice that had been vaccinated sc. For the groups of mice that had been immunized with only one dose sc., low levels of *M. tuberculosis* H37Rv $\Delta RD1$ bacilli were recovered from the spleen after 28 and 56 days post-vaccination; however, no splenic mycobacteria were detected 84 days after the sc. injection. Importantly, the concentration of *M. tuberculosis* H37Rv $\Delta RD1$ organisms in the lungs after the sc. immunizations was below the threshold of detection (<100 CFUs per organ) for the CFU assay at nearly all time points during the three month study.

Table 1. Growth kinetics in C57BL/6 mice.

Weeks	Lung (Log CFU)			Spleen (Log CFU)		
	i.v.	s.c.	s.c. (2X)	i.v.	s.c.	s.c. (2X)
4	5.86±0. 10	<2	not done	5.73±0. 05	2.41±0. 26	not done
8	5.79±0. 07	<2	2.52±0. 34	5.37±0. 04	3.12±0. 40	3.62±0. 29
12	5.61±0. 09	<2	<2	5.40±0. 05	<2	3.52±0. 22

Mice were infected with 10^6 *M. tuberculosis* H73Rv $\Delta RD1$ by different routes. The data are presented as mean \pm standard error of the mean.

Three months after the sc. vaccinations with the $\Delta RD1$ strain, groups of mice were challenged aerogenically with a low dose (50 CFUs) of an acriflavin-resistant strain of *M. tuberculosis* Erdman. The use of a drug-resistant challenge strain permitted the differentiation of the challenge organisms from the sensitive vaccine population. As controls, other groups of mice were immunized sc. with 10^6 CFUs of BCG Pasteur. The protective responses induced by the *M. tuberculosis* H37Rv $\Delta RD1$ vaccination were evaluated by assessing the relative growth of the acriflavin-resistant challenge organisms in naïve, BCG vaccinated, and *M. tuberculosis* H37Rv $\Delta RD1$ immunized mice and by comparing the relative post-challenge lung pathology in the experimental groups and the naïve controls. As seen in Table 2, the growth of the drug-resistant challenge organisms was substantially lower in the lungs of animals vaccinated with BCG or the *M. tuberculosis* H37Rv $\Delta RD1$ vaccine. Significant reductions in the lung CFU values in the vaccinated animals (relative to naïve controls) could be detected both 28 and 56 days after the challenge. Dissemination to the spleen was also significantly limited in all of the vaccination groups with the most substantial differences ($-1.4 \log_{10}$ CFUs compared to the naïves) being detected

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during the first month post-challenge. While significant differences in the growth of the mycobacterial challenge was identified between unvaccinated and vaccinated mice, the rate of proliferation of the acriflavin-resistant challenge strain in all the experimental groups (BCG sc or *M. tuberculosis* H37Rv $\Delta RD1$ 1 or 2 doses sc) was nearly identical and not statistically different.

Table 2. *M. tuberculosis* $\Delta RD1$ and BCG protect C57BL/6 mice from aerosol challenge with *M. tuberculosis* Erdman

	Lung (Log CFU)		Spleen (Log CFU)	
	Day 28	Day 56	Day 28	Day 56
Naive	4.77 \pm 0.06	4.11 \pm 0.05	3.57 \pm 0.21	3.20 \pm 0.16
BCG (1X)	3.96 \pm 0.20	3.80 \pm 0.08	2.18 \pm 0.18	2.48 \pm 0.23
$\Delta RD1$ (1X)	3.97 \pm 0.39	3.71 \pm 0.06	2.12 \pm 0.12	2.60 \pm 0.25
$\Delta RD1$ (2X)	3.96 \pm 0.15	3.66 \pm 0.09	2.21 \pm 0.15	2.22 \pm 0.16

Immunizations were performed subcutaneously once (1X) or twice (2X) with 2×10^6 CFUs of the vaccinating strains. Three months later, vaccinated animals were aerogenically challenged with 50 CFUs/mouse of acriflavin resistant *M. tuberculosis* Erdman. The growth of the bacterial challenge was monitored 28 and 56 days post infection by plating on Middlebrook 7H11 plates containing 20 μ g/ml acriflavin and using procedures previously described (Delogu et al., 2002).

Discussion

The *M. tuberculosis* H37Rv $\Delta RD1$ mutant strain shares significant properties with BCG including: 1) a significant attenuation of virulence in mice, 2) the ability to generate variable colonial morphotypes, and 3) the ability to protect mice against aerogenic tuberculosis challenge. These properties, and the observation that *RD1* is the only deletion common to all BCG substrains, makes it likely that the *RD1* deletion is the primary attenuating mutation. It remains to be determined if a single gene or a number of genes in this region causes the attenuated phenotype. The variable colonial morphotype switch does suggest that a protein regulating cell wall biogenesis is affected. Notably, defined mutations affecting the cyclopropanation of mycolic acids (Glickman et al., 2000) or the synthesis or export of phthiocerol dimycoserate (Cox et al., 1999) have been found to correlate with decreased virulence and altered colony morphotypes in *M. tuberculosis* and thus represent attractive candidate genes that might be regulated by an *RD1*-encoded gene. The *M. tuberculosis* $\Delta RD1$

mutant provides a precisely defined background strain by which to determine virulence and colony morphology related genes.

BCG is currently the only antituberculous vaccine available for use in humans. In many animal models, BCG has been shown to induce protective immunity against *M. tuberculosis* challenge (Opie and Freund, 1937; Hubbard et al., 1992; Baldwin et al., 1998) and in addition, has demonstrated protection against the most severe and fatal form of TB in children (Rodrigues et al., 1991). However, BCG has shown variable efficacy in protecting adults from pulmonary TB (Tuberculosis Prevention Trial, 1980; Hart and Sutherland, 1977; Bloom and Fine, 1994). Due to the uncertain efficacy of BCG, particularly in TB-endemic countries, the development of improved tuberculosis vaccines has become an international research priority.

Our challenge studies have demonstrated that the protective immune responses elicited by immunization with *M. tuberculosis* H37Rv $\Delta RD1$ in mice are at least as strong as the protective responses induced by vaccination with BCG. The *M. tuberculosis* H37Rv $\Delta RD1$ mutant also retains the BCG-associated property of limited spread to the lung following subcutaneous immunization. Restricted dissemination of the $\Delta RD1$ mutant to the lung suggests it should have a favorable overall safety profile. Also, the unmarked mutant of *M. tuberculosis* H37Rv $\Delta RD1$ provides a single deletion strain whereby other attenuating mutations can be readily engineered. Since the risk of reversion to wild-type virulence decreases substantially with each additional attenuating mutation, *M. tuberculosis* mutants harboring deletions in two or three separate genetic loci should provide a much safer vaccine for long term use.

M. tuberculosis mutants with *RD1* deletions represent attractive candidates as novel vaccines for TB prevention. These mutants, derived from a single mutagenic event from the parental *M. tuberculosis* strain, replicate more efficiently *in vivo* than BCG, especially early in infection. This enhanced rate of proliferation for the *RD1*-deleted strains, relative to BCG, may lead to the induction of increased protective immunity in humans, after vaccination with *M.*

tuberculosis H37Rv $\Delta RD1$. Moreover, they could also be more immunogenic as there exist at least 129 ORFs present in *M. tuberculosis* H37Rv that are absent from *M. bovis* (Behr et al., 1999). Since some of these ORFs are likely to encode regulatory proteins affecting the expression of other genes, there could be hundreds of antigens expressed in *M. tuberculosis*-infected cells that are absent from BCG-infected cells. Thus, *RD1* deletion mutants constructed from human tubercle bacilli could protect humans against disease substantially better than BCG.

Example 2. Vitamin auxotrophs of *Mycobacterium tuberculosis* are attenuated and protect against tuberculosis

This example describes experimental methods and results that establish that deleting genes that control vitamin production in a virulent *M. tuberculosis* causes the *M. tuberculosis* to become avirulent and sustain an infection in mammals, and protect the mammal against challenge with a virulent *M. tuberculosis*.

Given the importance of NAD and nicotinamide (vitamin B3) and pantothenate (vitamin B5) as cofactors involved in carbon utilization, energy transduction (Abiko, 1975; Jackowski, 1996) and the biosynthesis of the complex lipid cell wall of *M. tuberculosis*, we hypothesized that mutations in the biosynthetic pathways for NAD and pantothenate could lead to the generation of mutant strains that retain a limited ability to replicate and subsequently get cleared within the host tissues. In *M. tuberculosis*, the *nadABC* operon controls the *de novo* biosynthesis of NAD. Similarly, the *panC* and *panD* genes that are organized in an operon control the rate-limiting step in the *de novo* biosynthesis of pantothenate. We constructed deletion mutants of *M. tuberculosis* in the *nadBC* and *panCD* genes using specialized transduction, as described in Example 1. The mutant strains mc²3122 ($\Delta nadBC$) and mc²6001 ($\Delta panCD$) were auxotrophic for nicotinamide and pantothenate respectively. The *in vitro*

reversion frequencies of the respective mutations were found to be less than 10^{-10} events per generation.

The safety and attenuation of $\Delta nadBC$ and $\Delta panCD$ auxotrophic mutants were assessed by infection of immune-compromised SCID mice. SCID mice infected with wild-type *M. tuberculosis* and the $\Delta nadBC$ mutant succumbed to infection in about 5 weeks (data not shown). This result clearly indicates that in the absence of T-cell immunity, intermediates of NAD biosynthetic pathway, such as nicotinamide, are readily available in the macrophages to support the growth of the $\Delta nadBC$ mutant. In contrast all mice infected with the $\Delta panCD$ mutant survived longer than 30 weeks, demonstrating the severe attenuation of this mutant strain. The full virulence phenotype was restored when the *panCD* wild type alleles were integrated into the chromosome of the $\Delta panCD$ mutant in single copy, suggesting the observed attenuation in $\Delta panCD$ to be due to the requirement of pantothenate for growth and not due to polar effects of the mutation on downstream genes. SCID mice infected with the same dose of conventional BCG-Pasteur vaccine strain succumbed to infection within 80 days (Fig. 6A) in accordance with earlier reports (Guleria, 1996). Enumeration of bacterial burdens in SCID mice infected with wild type *M. tuberculosis* H37Rv and the complementing strain (*panCD* in single copy integrated into the chromosome) showed a rapid increase in bacterial numbers in spleen, liver and lung before they succumbed to infection. In contrast, mice infected with $\Delta panCD$ mutant, showed an initial drop in bacterial numbers in spleen and liver followed by a steady increase to reach 10^8 in the lungs at 160 days, at which time all mice were still alive (Fig. 6B).

Having demonstrated the significant attenuation of $\Delta panCD$ mutant, we sought to address the *in vivo* growth characteristics of this mutant in immune-competent BALB/c mice. All BALB/c mice infected with H37Rv succumbed to infection by day 25 with a MST of 22 days. Similarly, mice infected with the *panCD*-complemented strain were highly virulent with 100% mortality between 3-8 weeks post-infection similar to the wild type strain, with a MST of 28 days.

In contrast, all mice infected with $\Delta panCD$ mutant survived for over 33 weeks demonstrating the severe attenuation phenotype of this mutant in immune-competent mice (Fig. 7A). Interestingly, bacterial enumeration at three weeks post infection showed 1 log increase in the $\Delta panCD$ numbers in lungs followed by a state of persistence with the onset of adaptive immune response. This growth characteristic was observed only in the lung but not in spleen or liver (Fig. 7B,C). A desirable trait of an effective live attenuated vaccine strain is its ability to grow within the host in a limited fashion in order to produce *in vivo* all the important protective antigens (McKenney, 1999; McKenny, 2000; Kanai, 1955). The $\Delta panCD$ mutant exhibits this characteristic in the lung, which is the primary site of infection in humans and does not get cleared over a prolonged period in all the three organs. The earlier auxotrophs of *M. tuberculosis* failed to grow in any of the organs and hence failed to adequately protect against experimental challenge in guinea pigs (Jackson, 1999), or mice.

The ability of the $\Delta panCD$ mutant to exhibit limited growth in the lung until the onset of adaptive immune response suggests that an unidentified putative pantothenate permease is able to transport this nutrient into resting macrophages, as in the SCID mice. A sodium-dependent pantothenate permease actively transports pantothenate into the cell of *Escherichia coli* (Vallari and Rock, 1985; Jackowski and Alix, 1990), *Plasmodium falciparum* (Saliba and Kirk, 2001) and mammals. Subsequent activation of macrophages leads to restricted supply of this nutrient within the phagosome resulting in growth arrest of the mutant. Pantothenic acid or its derivatives have been reported to confer resistance to radiation and oxidative stress by virtue of their role in biosynthesis of CoA and also by indirectly increasing the cellular supply of glutamate, a precursor of glutathione (Slyshenkov, 1995). Pantothenate kinase (*PanK*) mutants of *Drosophila* display membrane defects and improper mitosis and meiosis due to decreased phospholipid biosynthesis (Afshar et al., 2001). The disruption of *de novo* pantothenate biosynthesis causes an increased

susceptibility of the $\Delta panCD$ mutant to reactive oxygen and nitrogen intermediates that are released within activated macrophages.

Having observed the $\Delta nadBC$ mutant to be non-attenuated in SCID mice, we chose to study the *in vivo* growth kinetics of this mutant in the more resistant C57BL/6 mice background. During the first three weeks of infection, the number of wild type and mutant bacteria recovered from all three organs showed little or no difference. Their numbers gradually increased in the lungs to reach 10^6 . However, with the onset of adaptive immune response at three weeks, when the growth of bacteria in the lungs of mice infected with H37Rv became constant and tightly controlled, bacterial load in the lungs of mice infected with $\Delta nadBC$ mutant showed a constant tendency for clearance to reach more than 1.5 log drop in the bacterial numbers compared to mice infected with wild type strain (Fig. 8A). This difference was preserved up to 24 weeks following infection.

The reduced ability of the $\Delta nadBC$ mutant to sustain an infection was accompanied by attenuated virulence clearly seen from the survival experiment (Fig. 8C). While all mice infected with the wild type strain succumbed to infection between day 90 and 179 (MST 116 days) all mice infected with the $\Delta nadBC$ mutant (n=12) remain alive for a period of more than 8 months (Fig. 8C).

Our observation of the attenuation phenotype of $\Delta nadBC$ mutant became obvious only after the onset of immune response, suggesting that once the macrophages become activated, they restrict the amount of available NAD or NAD intermediates causing a restricted growth of the mutant strain. This would be in agreement with the recently reported observations that a significant part of antimicrobial function of the macrophages could be attributed to the IFN- γ promoted enhanced expression of indolamine 2-oxygenase (IDO), the inducible enzyme controlling L-tryptophan catabolic pathway causing an almost complete depletion of L-tryptophan pool. The enhanced catabolism of L-tryptophan leads to increased *de novo* biosynthesis of NAD needed to protect the cells from the

free radicals formed as a result of macrophage activation. Recently, several studies have demonstrated the involvement of the tryptophan catabolism in the antimicrobial mechanisms of the activated macrophages. Induction of IDO was found responsible for the inhibition of intracellular growth of *Toxoplasma*, *Leishmania*, *Legionella* and *Chlamydia*. The restricted intracellular growth of $\Delta nadBC$ mutant could be explained with the very little amount of free NAD or NAD intermediates available within the activated macrophages.

Having established the safety and persistence of $\Delta panCD$ and $\Delta nadBC$ in immunocompetent mice, the protective efficacy of these mutants were evaluated using an aerosol challenge model with virulent *M. tuberculosis*, using the methods described in Example 1. The aerosol route of infection was chosen, as this is the natural route of infection in humans. To assess the capacity of the auxotrophic vaccines to restrict growth of virulent *M. tuberculosis* in the organs of infected mice, bacterial numbers were enumerated one month post-infection in lung and spleen. See Table 3. In the unimmunized controls, bacterial numbers rose rapidly in the spleen and lungs, in contrast mice infected with a single dose of $\Delta panCD$ displayed significant reduction in bacterial numbers in the spleen and lung ($p < 0.05$, in comparison to unimmunized controls). Mice given two doses of $\Delta panCD$ displayed a statistically significant reduction in the bacterial numbers to 4.5 log units in the lung ($p < 0.01$) and 3.7 log units in the spleen ($p < 0.05$). Mice vaccinated with BCG showed comparable reduction in bacterial burden in the lung and spleen to 3.3 log units and 4.7 log units respectively ($p < 0.01$). Mice immunized with one or two doses of $\Delta nadBC$ mutant conferred statistically significant protection ($p < 0.01$ in comparison to unimmunized group) that is comparable to the protection afforded by BCG vaccination. Interestingly, mice immunized with the $\Delta nadBC$ mutant showed no detectable CFUs in the spleen suggesting that the vaccination completely prevented the hematogenous spread of wild type *M. tuberculosis* following aerosol challenge.

Table 3.

A		
Experimental Group	Lung CFUs (\log_{10})	Spleen CFUs (\log_{10})
Naive	4.05 ± 0.21	3.94 ± 0.21
$\Delta nadBC$ (1 x sc)	$3.37 \pm 0.40^{**}$	$<2^{**}$
$\Delta nadBC$ (2 x sc)	$3.6 \pm 0.35^{**}$	$<2^{**}$
BCG (1 x sc)	$3.46 \pm 0.19^{**}$	$<2^{**}$

B		
Experimental Group	Lung CFUs (\log_{10})	Spleen CFUs (\log_{10})
Naive	5.56 ± 0.05	4.35 ± 0.21
$\Delta panCD$ (1 x sc)	$4.99 \pm 0.17 (-0.57)^*$	$3.65 \pm 0.15 (-0.70)^*$
$\Delta panCD$ (2 x sc)	$4.55 \pm 0.09 (-1.01)^{**}$	$3.73 \pm 0.21 (-0.62)^*$
BCG (1 x sc)	$4.71 \pm 0.21 (-0.85)^{**}$	$3.35 \pm 0.20 (-1.00)^{**}$

$p < 0.05$ compared to naïve, $^{**} p < 0.01$ compared to naïve

Table 3. The attenuated *M. tuberculosis* $\Delta nadBC$ and $\Delta panCD$ mutants protect against aerogenic challenge with *M. tuberculosis* Erdman. Groups of C57BL/6 mice (5 mice per group) were vaccinated subcutaneously either once or twice (6 weeks apart) with 10^6 CFUs of mutant strains. Control mice were vaccinated subcutaneously with 10^6 CFUs of BCG-Pasteur. Three months after the initial immunization with either $\Delta nadBC$ or $\Delta panCD$ mutant or BCG, the mice were aerogenically challenged with approximately 100 CFUs of acriflavin-resistant *M. tuberculosis* Erdman (Ac^rMTB) strain as described earlier (Collins, 1985). After 28 days, the challenged mice were sacrificed, and the lungs and spleens of individual mice were removed aseptically and homogenized separately in 5 ml of Tween 80-saline using a Seward stomacher 80 blender (Tekmar, Cincinnati, OH). The homogenates were diluted serially in Tween 80 saline and plated on Middlebrook 7H11 agar with or without appropriate supplements as required. Samples from the BCG-vaccinated controls were plated on 7H11 agar containing 2 mg of thiophenecarboxylic acid hydrazide (Sigma Chemical Co., St Louis, MO) per ml to inhibit growth of any residual BCG. The CFU results were evaluated using the one-way ANOVA analysis of the Graph Pad InStat program. The numbers in paranthesis represent the differences between naïve and vaccinated organ CFUs.

In order to test the ability of the auxotrophic mutants to confer long lasting immunity, mice were challenged 7 months after an initial subcutaneous immunization with the $\Delta nadBC$ mutant. See Table 4. Mice immunized with

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ΔnadBC displayed significantly reduced numbers of the challenge organism in the lungs and no detectable numbers in the spleen comparable to the numbers seen in the BCG vaccinated mice. The results suggest that the *ΔnadBC* vaccine strain is able to persist within the mouse organs sufficiently long to mount a long lasting immunity to control subsequent infection.

Table 4.

Experimental Group	Lung CFUs (\log_{10})	Spleen CFUs (\log_{10})
Naive	4.61 ± 0.07	4.07 ± 0.20
BCG	$4.00 \pm 0.13^*$	2
NAD (1 x iv)	$3.28 \pm 0.15^{**}$	<2
NAD (2 x iv)	$2.95 \pm 0.14^{**}$	<2
NAD (1 x sc)	$4.05 \pm 0.12^*$	<2
NAD (2 x sc)	$3.94 \pm 0.13^*$	<2

* $P < 0.05$; ** $P < 0.01$ by Dunnett's Multiple Comparison Test

Table 4. Immunizations with the $\Delta nadBC$ mutant confer long-term protection against an aerosol challenge. Groups of C57BL/6 mice (5 mice per group) were vaccinated subcutaneously or intravenously either once or twice (6 weeks apart) with 10^6 CFUs of $\Delta nadBC$ mutant. Control mice were vaccinated subcutaneously with 10^6 CFUs of BCG-Pasteur. Seven months after the initial immunization with either $\Delta nadBC$ mutant or BCG, the mice were aerogenically challenged with approximately 50 CFUs of acriflavin-resistant *M. tuberculosis* Erdman (Ac^rMTB) strain and the bacterial numbers at 28 days post challenge enumerated as described in Table 1.

To the best of our knowledge this is the first report of any *M. tuberculosis* auxotrophic vaccines administered subcutaneously to confer protection comparable to the conventional BCG vaccine strain in a mouse model of infection. Mice vaccinated with the $\Delta panCD$ and $\Delta nadBC$ survived for over one year following the aerosol challenge indicating the protection and safety of these vaccine strains.

Example 3. A pantothenate auxotroph of *Mycobacterium tuberculosis* is highly attenuated and protects mice against tuberculosis.

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Example summary.

With the advent of HIV and the widespread emergence of drug resistant strains of *Mycobacterium tuberculosis*, newer control strategies in the form of a better vaccine could decrease the global incidence of tuberculosis. A desirable trait in an effective live attenuated vaccine strain is its ability to persist within the host in a limited fashion in order to produce important protective antigens

in vivo (Kanai and Yanagisawa, 1955; McKenney et al., 1999). Rationally attenuated *M. tuberculosis* vaccine candidates have been constructed by deleting genes required for growth in mice (Jackson et al., 1999; Hondalus et al., 2000; Smith et al., 2001). These candidate vaccines failed to elicit adequate protective immunity in animal models, due to their inability to persist sufficiently long within the host tissues. Here we report that an auxotrophic mutant of *M. tuberculosis* defective in the *de novo* biosynthesis of pantothenic acid (vitamin B5) is highly attenuated in immunocompromised SCID mice and in immunocompetent BALB/c mice. SCID mice infected with the pantothenate auxotroph survived significantly longer than mice infected with either BCG vaccine or virulent *M. tuberculosis* (250 days, vs. 77 days, vs. 35 days). Subcutaneous immunization with this auxotroph conferred protection in C57BL/6J mice against an aerosol challenge with virulent *M. tuberculosis*, which was comparable to that afforded by BCG vaccination. Our findings highlight the importance of *de novo* pantothenate biosynthesis in limiting the intracellular survival and pathogenesis of *M. tuberculosis* without reducing its immunogenicity in vaccinated mice.

Materials and Methods.

Media and Strains. *M. tuberculosis* H37Rv, *M. tuberculosis* Erdman and *M. bovis* BCG Pasteur were obtained from the Trudeau Culture Collection (Saranac Lake, NY) and cultured in Middlebrook 7H9 broth and 7H11 agar supplemented with 10% OADC, 0.5% glycerol, and 0.05% Tween 80. When required, pantothenate (24 µg/ml), hygromycin (50 µg/ml) or kanamycin (25 µg/ml) was added.

Stock strains were grown in Middlebrook 7H9 broth in roller bottles and harvested in mid-logarithmic growth phase, before being stored in 1 ml vials at -70° C until required.

Disruption of *panCD* genes in *M. tuberculosis*. Specialized transduction was employed to disrupt the chromosomal copy of the *panCD* genes as described (U.S. Patent 6,271,034). Briefly, the 823 bp region upstream to the *panC* gene was amplified using primers Pan1 (5'-GTGCAGCGCCATCTCTCA-3') and Pan2

(5'-GTTACACGGGATGGAACG-3'). A 716 bp region downstream to the *panD* gene was amplified using primers Pan3 (5'-CCCGGCTCGGTGTGGGAT-3') and Pan4 (5'-GCGCGGTATGCCCGGTAG-3'). PCR products were cloned with the TOPO TA cloning kit (Invitrogen, CA), and sequenced. PCR products were subsequently cloned into pJSC347, flanking a hygromycin cassette to create pSKV1. *PacI* digested pSKV1 was ligated into the temperature-sensitive mycobacteriophage phAE159 derived from TM4 and transduced as described earlier (Glickman et al., 2000; Raman et al., 2001). Genomic DNAs from hygromycin-resistant and pantothenate-requiring colonies were digested with *Bss*III, and probed with a 716 bp downstream region, flanking the *M. tuberculosis panCD* operon to confirm the deletion. For complementation, the *M. tuberculosis panCD* operon was amplified by PCR from genomic DNA with its putative promoter, cloned with TA cloning kit, sequenced, and subcloned into pMV306kan, a site-specific integrating mycobacterial vector.

Animal infections. C57BL/6, BALB/cJ and BALB/c SCID mice (6-8 weeks old) were purchased from Jackson Laboratories and were infected intravenously through the lateral tail vein. For time-to-death assays, BALB/c SCID mice were infected intravenously with 1×10^2 CFU of *M. tuberculosis* H37Rv, 1×10^2 CFU of *panCD*-complemented strain, 1×10^5 CFU of Δ *panCD* mutant, or 1×10^5 CFU of *M. bovis* BCG-P. For mouse organ CFU assays, BALB/cJ mice were infected with 1×10^6 CFU of *M. tuberculosis* H37Rv or the *panCD*-complemented strain or the Δ *panCD* mutant. At appropriate time points, groups of 4-5 mice were sacrificed and the selected organs were homogenized separately in PBS/0.05% Tween 80, and colonies were enumerated on 7H11 plates grown at 37° C for 3-4 weeks (see McKinney et al., 2000). Pathological examination was performed on tissues fixed in 10% buffered formalin. The CFU results were evaluated using the one-way ANOVA analysis of the Graph Pad InStat program. All animals were maintained in accordance with protocols approved by the Albert Einstein College of Medicine Institutional Animal Care and Use Committee.

Vaccination Studies. Groups of C57BL/6 mice (5 mice per group) were vaccinated subcutaneously either once or twice (6 weeks apart) with 1×10^6 CFU of the $\Delta panCD$ mutant strain. Control mice were vaccinated subcutaneously with 1×10^6 CFU of *M. bovis* BCG-P. Three months after the initial immunization with either the $\Delta panCD$ mutant or BCG, the mice were aerogenically challenged with approximately 50-100 CFU of *M. tuberculosis* Erdman strain as described earlier. At 28 days following aerosol challenge, the challenged mice were sacrificed; the lungs and spleens of individual mice were removed aseptically and homogenized separately in 5 ml of Tween 80-saline using a Seward Stomacher 80 blender (Tekmar, Cincinnati, Ohio). The homogenates were diluted serially in Tween 80 saline and plated on Middlebrook 7H11 agar with or without appropriate supplements as required. Samples from the BCG-vaccinated controls were plated on 7H11 agar containing 2 mg/ml of thiophene-2-carboxylic acid hydrazide (Sigma) to inhibit growth of any residual BCG.

Results and Discussion.

Lipid biosynthesis and metabolism have been shown to play a pivotal role in the intracellular replication and persistence of *M. tuberculosis* (Cox et al., 1999; Camacho et al., 1999; Glickman et al., 2000; De Voss et al., 2000; Manca et al., 2001; McKinney et al., 2000). Therefore, we sought to globally impair the ability of this bacterium to synthesize lipids. Pantothenic acid (vitamin B5) is an essential molecule required for the synthesis of coenzyme A (CoA) and acyl carrier protein (ACP), that play important roles as acyl group carriers in fatty acid metabolism, the tricarboxylic acid cycle, biosynthesis of polyketides and several other reactions associated with intermediary metabolism (Jackowski, 1996). Bacteria, plants and fungi synthesize pantothenate from amino acid intermediates, whereas it is a nutritional requirement in higher animals (FIG 9a).

We constructed a double deletion mutant of *M. tuberculosis* in the *panC* and *panD* genes that are involved in the de novo biosynthesis of pantothenate

(FIG. 9b,c). The $\Delta panCD$ mutant was found to be auxotrophic for pantothenate with no detectable reversion to prototrophy when 1×10^{10} cells were plated on minimal medium. The growth rate of the mutant was identical to wild type H37Rv in broth cultures in the presence of exogenous pantothenate (data not shown). The attenuation of the $\Delta panCD$ mutant was assessed by infection of immunocompromised SCID mice. SCID mice infected intravenously with H37Rv succumbed to the resulting infection in about 5 weeks. In contrast, all mice infected with the $\Delta panCD$ mutant survived for more than 36 weeks (average, 253 days) (FIG. 10a). This attenuation is due to pantothenate auxotrophy as the full virulence phenotype was restored when the *panCD* wild type genes were integrated into the chromosome of the $\Delta panCD$ mutant in single copy. Enumeration of bacterial burdens in SCID mice infected with H37Rv and the $\Delta panCD$ -complemented strain showed a rapid increase in bacterial numbers in the spleen, liver and lung, until they succumbed to infection. In contrast, mice infected with the $\Delta panCD$ mutant showed an initial drop in bacterial numbers in the spleen and liver followed by a gradual increase in the number of viable bacteria, reaching 1×10^6 colony-forming units (CFU) by day 224 (FIG. 10b). Notably, the CFU values increased to 1×10^8 in the lungs of the infected mice. The ability of $\Delta panCD$ -infected SCID mice to survive despite a substantial bacterial burden in their lungs emphasizes the extent of attenuation in this mutant and compares with the phenotype observed with the *M. tuberculosis whiB3* and *sigH* mutants described recently (Steyn et al., 2000; Kaushal, 2000). Notably, SCID mice infected with bacille Calmette-Guerin-Pasteur (BCG-P) strain succumbed to infection by 83 days (Weber et al., 2000) in contrast to the prolonged survival observed in $\Delta panCD$ -infected mice.

Studies in immunocompetent mice further demonstrate the attenuation of the $\Delta panCD$ mutant. Survival studies showed that BALB/c mice infected with H37Rv succumbed to infection by day 25 (average, 21 days) and mice infected with an identical dose of the *panCD*-complemented strain succumbed to infection between days 21 to 53 (average, 37 days). Importantly, all mice

infected with 1×10^6 CFU of the $\Delta panCD$ mutant survived 375 days, when the experiment was terminated (FIG. 10c). At 3 weeks post infection, in contrast to the H37Rv strain, BALB/c mice infected with $\Delta panCD$ mutant showed a 10-fold increase in bacterial numbers in the lungs followed by a gradual decline in viable numbers over the next 38 weeks of infection (FIG. 10d) and the bacterial burden gradually declined in the spleen and liver throughout the course of infection (FIG. 10e). Histopathologic examination of the lungs from mice infected with either H37Rv or the $\Delta panCD$ -complemented strain, showed severe, diffuse lobar granulomatous pneumonia (FIG. 11a,b). The pneumonia affected more than 50% of the lung, and was pyogranulomatous with marked necrosis in the advanced consolidated areas, particularly in the lungs of mice challenged with H37Rv. Both of these strains caused severe granulomatous splenitis and widespread granulomatous hepatitis. At 3 weeks post-infection with the $\Delta panCD$ mutant, low to moderate numbers of focal infiltrates of mononuclear cells scattered throughout the lung were seen (FIG. 11c). The spleen was moderately enlarged with scattered granulomas. Similarly, the liver showed numerous focal granulomas. At 24 weeks post-infection, consistent with the bacterial numbers, histological examination of the lungs from mice infected with the $\Delta panCD$ mutant showed only occasional focal mild infiltrations, predominately lymphocytic (FIG. 11d). The spleen showed only mild histiocytic hyperplasia and there were fewer, focal, predominately lymphocytic accumulations in the liver.

The mechanisms that allow the persistence of the $\Delta panCD$ mutant bacteria for over 8 months in the SCID mouse model remain unclear. We speculate the functional role of an unidentified permease in transporting adequate amount of pantothenate in the $\Delta panCD$ mutant that allows its persistence but not the ability to cause disease. A pantothenate permease that transports pantothenate have been described in *Plasmodium falciparum* and *Escherichia coli* (Saliba and Kirk, 2001; Jackowski and Alix, 1990). In the lungs of immunocompetent mice, an initial growth of the $\Delta panCD$ mutant during the

first 3 weeks is followed by a steady decline in bacterial numbers following the onset of an adaptive immune response. The intracellular lifestyle of *M. tuberculosis* poses significant challenges to the bacterium in acquiring essential nutrients. Pantothenic acid or its derivatives have been shown to confer resistance to oxidative stress (Slyshenkov et al., 1996) and lack of pantothenate biosynthesis in the $\Delta panCD$ mutant may render it more susceptible to such adverse effects. Likewise, a pantothenate kinase (*panK*) mutant of *Drosophila* was shown to display membrane defects and improper mitosis and meiosis due to decreased phospholipid biosynthesis (Afshar et al., 2001). Therefore, it is plausible that the pantothenate salvage pathway is inadequate in restoring full virulence of the $\Delta panCD$ mutant in the absence of a functional de novo biosynthetic pathway.

As a test of vaccine potential, immunized mice were challenged with virulent *M. tuberculosis* Erdman by the aerosol route (Collins, 1985). Following subcutaneous immunization, the $\Delta panCD$ mutant could not be detected in the spleens or lungs of mice at 8 and 12 weeks. In the naive controls, the bacterial CFU values increased 10,000-fold in the lung during the first month after challenge. Similarly, substantial dissemination and growth in the spleen was detected within one month of the challenge in naive controls. In contrast, mice immunized with single or double doses of the $\Delta panCD$ mutant displayed statistically significant reductions ($P < 0.05$) in lung and spleen CFU values relative to naive controls. Mice vaccinated with BCG showed similar reduction in organ bacterial burdens compared to the nonimmunized controls (FIG. 11e,f). In these aerogenic challenge studies, no significant differences were detected in the lung and spleen CFU values for mice vaccinated with either the $\Delta panCD$ mutant strain or with BCG. At 28 days after the aerogenic challenge with virulent *M. tuberculosis*, histopathological examination of lungs of $\Delta panCD$ immunized mice revealed a less severe infection relative to the unvaccinated control mice. In controls, severe bronchitis, moderate pneumonia, and spread of the infection to the adjacent lung parenchyma was observed. By comparison,

the $\Delta panCD$ vaccinated mice had milder bronchitis and smaller areas of mild interstitial pneumonitis, with localized areas of granulomatous pneumonia in some mice. Importantly, no lung pathology was detected in vaccinated mice at the time of challenge (data not shown). Two groups of mice that were vaccinated with one or two doses of the $\Delta panCD$ mutant and then challenged with *M. tuberculosis* Erdman were active and healthy for more than one year following the virulent challenge. Histopathological analysis of lung sections from these mice showed only mild inflammation and fibrosis despite the chronic infection.

By creating a *M. tuberculosis* strain that is defective in pantothenate biosynthesis, we have taken a critical step in the rational development of an attenuated *M. tuberculosis* vaccine strain. We have shown that a functional pantothenate biosynthetic pathway, which is required for the synthesis of complex mycobacterial lipids, is essential for the virulence of *M. tuberculosis*. Although the precise mechanism of the reduced virulence is unclear, it is reasonable to speculate that this could be due to reduced synthesis of toxic polyketides and secreted lipids or a general slow down of metabolism. Tubercle bacilli lacking the two genes required to synthesize pantothenate failed to revert and were highly attenuated and less virulent than BCG vaccine when tested in the rigorous SCID mouse model of infection. Despite the reduced virulence associated with the deletion of the *panCD* genes, these vitamin auxotrophs remain persistent *in vivo* as shown by their ability to survive for at least eight months in immunocompetent mice. The persistence of this mutant strain undoubtedly contributes to the substantial immunogenicity seen in the mouse tuberculous challenge model. Overall, the $\Delta panCD$ mutant has many of the characteristics necessary for a live vaccine candidate strain: it is attenuated by a non-reverting mutation and essentially avirulent while being persistent and immunogenic. Given the genetic differences between *M. bovis* and *M. tuberculosis* (Behr et al., 1999), one would predict that a rationally attenuated *M. tuberculosis* strain would have a more relevant repertoire of species-specific

antigens and thus should elicit, in humans, more effective protective immune responses against tuberculous infections than BCG.

Example 4. The primary mechanism of attenuation of BCG is a loss of invasiveness due to host cell lysis.

Example Summary.

Tuberculosis remains a leading cause of death worldwide, despite the availability of effective chemotherapy and a vaccine. BCG, the tuberculosis vaccine, is an attenuated mutant of *M. bovis* that was isolated following serial subcultivations, yet the basis for this attenuation has never been elucidated. A single region (RD1), deleted in all BCG substrains, was deleted from virulent *M. bovis* and *M. tuberculosis* strains and the resulting three $\Delta RD1$ mutants were significantly attenuated for virulence in both immunocompromised and immunocompetent mice. Like BCG, *M. tuberculosis* $\Delta RD1$ mutants protect mice against aerosolized *M. tuberculosis* challenge and these mutants also consistently display altered colonial morphotypes. Interestingly, the $\Delta RD1$ mutants failed to cause necrosis, via lysis, of pneumocytes, a phenotype that had been previously used to distinguish virulent *M. tuberculosis* from BCG. We conclude that the primary attenuating mechanism of BCG is the loss of cytolytic activity, resulting in reduced invasiveness.

Introduction.

BCG (bacille Calmette and Guérin), was first isolated from *M. bovis* following serial subculturing of *M. bovis* in 1908 (Calmette and Guérin, 1909). Drs. Calmette and Guérin set out to test the hypothesis that a bovine tubercle bacillus could transmit pulmonary tuberculosis following oral administration (Calmette and Guérin, 1905; Gheorghiu, 1996) and developed a medium containing beef bile that enabled the preparation of fine homogenous bacillary suspensions. After the 39th passage, the strain was found to be unable to kill experimental animals (Calmette and Guérin, 1909). Between 1908 and 1921, the strain showed no reversion to virulence after 230 passages on bile potato

medium (Gheorghiu, 1996), which is consistent with the attenuating mutation being a deletion mutation. In the animal studies that followed, BCG was shown to be attenuated, but it also protected animals receiving a lethal challenge of virulent tubercle bacilli (Calmette and Guerin, 1920). BCG was first used as a vaccine against tuberculosis in a child in 1921 (Weill-Halle and Turpin, 1925). From 1921 to 1927, BCG was shown to have protective efficacy against TB in a study on children (Id.; Calmette and Plotz, 1929) and was adopted by the League of Nations in 1928 for widespread use in the prevention of tuberculosis. By the 1950's, after a series of clinical trials, the WHO was encouraging widespread use of BCG vaccine throughout the world (Fine and Rodrigues, 1990). Although an estimated 3 billion doses have been used to vaccinate the human population against tuberculosis; the mechanism that causes BCG's attenuation remains unknown.

Mahairas et al. (1996) first compared the genomic sequences of BCG and *M. bovis* using subtractive hybridization and found that there were three Regions of Difference (designated *RD1*, *RD2*, and *RD3*) present in the genome of *M. bovis*, but missing in BCG. Behr et al. (Behr et al., 1999) and others (Gordon et al., 2001) later identified 16 large deletions, including *RD1* to *RD3*, present in the BCG genome but absent in *M. tuberculosis*. Eleven of these 16 deletions were unique to *M. bovis*, while the remaining 5 deletions were unique to BCG. One of these 5 deletions, designated *RD1* (9454 bp), was absent from all of the BCG substrains currently used as TB vaccines worldwide and it was concluded that the deletion of *RD1* appeared to have occurred very early during the development of BCG, probably prior to 1921 (Behr et al., 1999). It is reasonable to hypothesize that *RD1* was the primary attenuating mutation first isolated by Calmette and Guerin to generate BCG from *M. bovis*. Attempts to restore virulence to BCG with *RD1*-complementing clones have been unsuccessful (Mahairas et al., 1996).

Results.

RD1 deletions of *M. bovis* and *M. tuberculosis* are attenuated for virulence in

immunocompromised mice. To test if *RD1* is essential for virulence in *M. bovis* and *M. tuberculosis*, it was necessary to delete the *RD1* (FIG. 1a) from virulent strains, demonstrate loss of virulence, and then restore virulence by complementation with the *RD1* DNA. Since the original *M. bovis* parent of BCG was lost in World War I (Grange et al., 1983), we initiated studies with virulent *M. bovis* Ravenel and a variety of virulent *M. tuberculosis* strains. Despite success in generating an unmarked deletion mutant of *RD1* in *M. tuberculosis* with a plasmid transformation system^{1,2}, over 100 independent transformations failed to yield an *RD1* deletion in *M. bovis*. As an alternative strategy, specialized transduction (Bardarov et al., 2002)³ was successfully used to generate *RD1* deletion mutants not only in *M. bovis* Ravenel, but also the H37Rv, Erdman, and CDC1551 strains of *M. tuberculosis* (FIG. 12). This deletion represents the largest deletion mutation generated by a targeted disruption in *M. tuberculosis* or *M. bovis* made to date and demonstrates the utility of the specialized transduction system. Moreover, since the parental specialized transducing phage has been shown to infect over 500 clinical *M. tuberculosis* isolates (Jacobs et al., 1987), it should be possible to introduce the *RD1* deletion into any *M. tuberculosis* or *M. bovis* isolate of interest.

To determine if the *RD1* deletion causes an attenuating phenotype in *M. bovis* and *M. tuberculosis*, the *M. tuberculosis* H37Rv $\Delta RD1$ was inoculated intravenously into immunocompromised mice possessing the SCID (severe combined immunodeficiency) mutation. Groups of ten mice were injected intravenously with either 2×10^6 wild type or $\Delta RD1$ strain of *M. tuberculosis* and *M. bovis*, and three mice per group were sacrificed 24 hours later to verify the inoculation doses. All of the SCID mice infected with the parental *M. tuberculosis* or *M. bovis* strain died within 14 to 16 days post-infection (FIG. 12A). In contrast, the SCID mice infected with equal doses of the $\Delta RD1$ strains of *M. tuberculosis* or *M. bovis* were all alive at 25 to 41 days post-infection, demonstrating a highly significant attenuation of the virulence of both strains. It is important to note that BCG-Pasteur kills SCID mice approximately 70 days

post-infection (FIG. 13B), suggesting that BCG substrains have acquired additional attenuating mutations which are consistent with the deletion analysis of BCG strains (Behr et al., 1999) and the previous failures to restore virulence with the RD1 region (Mahairas et al., 1996).

To prove that the attenuation of virulence was due to the *RD1* deletion, the *M. tuberculosis* $\Delta RD1$ was transformed with an integrating cosmid, 2F9, containing the *RD1* region from *M. tuberculosis* H37Rv⁴. SCID mice were infected as described above and the attenuation for virulence was restored to the parental virulent phenotype (FIG. 13B). These results strongly suggest that the genes deleted from the *RD1* region contribute to the virulence phenotype. The *M. tuberculosis* $\Delta RD1$ is highly attenuated in immunocompetent BALB/c mice. The virulence of the *M. tuberculosis* $\Delta RD1$ mutant was further assessed by intravenous inoculation of immunocompetent BALB/c mice. While the virulent parent *M. tuberculosis* strain killed the BALB/c mice in 10 to 17 weeks post-infections, 100% of mice were alive at 48 weeks and 43 weeks post-infections in two independent experiments (FIG. 13C).

While infection with BCG and *M. tuberculosis* $\Delta RD1$ yielded similar survival results in BALB/c mice, there were substantial differences in the growth kinetics in mice. BCG grew in a limited fashion in lungs, liver and spleen in BALB/c mice during the 22 weeks of the experiment (FIG. 4B-D). In contrast, the *M. tuberculosis* $\Delta RD1$ strain grew in a fashion indistinguishable from the parental *M. tuberculosis* H37Rv in all mouse organs for the first 8 weeks. Thereafter, mice infected with the parental *M. tuberculosis* failed to contain the infection leading to mortality. Strikingly, mice infected with the *M. tuberculosis* $\Delta RD1$ showed a definite control over infection resulting in significantly prolonged survival of mice (FIG. 4B-D).

Histopathological examination further demonstrated that the mutant was attenuated in virulence compared to the parent strain H37Rv (FIG. 5D-F). In contrast to the rapidly progressive infection with the parent strain, the lung lesions caused by the mutant were maximal in mice examined at 8 weeks

post-infection. Consolidating granulomatous pneumonia involved an estimated 25-30% of the lung in these mice. Numerous organisms were demonstrated by acid fast staining. The pneumonia subsequently underwent partial resolution. By 14 weeks, and again, at 22 weeks post-infection, the lungs showed peribronchial and perivascular inflammatory cell accumulations and focal, generally non-confluent, granulomas now with a prominent lymphocytes infiltration. The numbers of acid fast bacilli were reduced. Liver lesions consisted of low numbers of scattered granulomas. Spleens were smaller, with persistent granulomas in the red pulp. Mice infected with *M. bovis* BCG showed mild lesions in the lung, liver and spleen at all time points (FIG. 5G-I). At longer time intervals post-infection the lesions were fewer in number, and smaller with prominent lymphocytic infiltrations. At 14 weeks post-infection, *M. bovis* BCG was below the level of detection by acid fast staining. In summary, whereas *M. tuberculosis* $\Delta RD1$ initially grew in a manner similar to the parental *M. tuberculosis* H37Rv, this *RD1* mutant was limited in the extent of spread of infection, particularly in the lung. This contrasts the extensive and severe damage caused by the parent strain. The subsequent resolving granulomas, localization of the lesions and changes in the composition of the inflammatory cell infiltrations suggested that the mice mounted an effective immune response to combat *M. tuberculosis* $\Delta RD1$ infection and thereby reduced the numbers of viable organisms.

Early BCG properties: Altered colonial morphotypes and long-term immunogenicity. While frozen stocks of the original BCG strain do not exist, written records do exist describing the early BCG strains, as Dr. Calmette sent the strains to many laboratories. In a study published in 1929, Petroff and colleagues reported that BCG displayed two distinct colony types (Petroff et al., 1929). One morphotype was a smooth (S) phenotype that was flat and corded (like the parental virulent strain) and the second was a rough and raised (R) phenotype. The *M. tuberculosis* $\Delta RD1$ mutant was generated independently four times and consistently yielded a 20 to 50% mixture of two colonial

morphotypes on Middlebrook medium without Tween 80 (FIG. 3B). The distinction of these two types of morphology could be noted even when the colonies were less than two weeks old, as the rough colonies were constricted and elevated with only a small portion of the base of the colony attached to the agar, while the smooth colonies tended to be flattened and spread out. When colonies grew older, e.g. 6 weeks old, the rough colonies remained more constricted compared to those of smooth colonies. The rough colonies exhibited large folds on the surface (FIG. 3F-G), as compared to those of the smooth colonies that exhibited small wrinkles (FIG. 3E).

The generation of two distinct colonial morphotypes must be a phenotypic change induced by the deletion of *RD1*. The morphotypes could not be cloned, as repeated subculturing of either the R or S colonies continued to yield both colonial morphotypes. Moreover, Southern analysis of R and S colonies revealed each morphotype had the same *RD1*-deleted genotype (FIG. 3D). Furthermore, complementation of *M. tuberculosis* $\Delta RD1$ with the *RD1* region restored the mutant phenotype back to the homogenous parental S phenotype (FIG. 3A-C). These results suggest that the variable morphotypes resulted directly from the *RD1* deletion. It can therefore be postulated that a regulator of colonial morphology is affected by one or more of the deleted genes.

One of the hallmark characteristics of BCG is its ability to provide protection against aerosolized challenge with virulent *M. tuberculosis*. To test the potential of *M. tuberculosis* $\Delta RD1$ to immunize and protect mice against tuberculous challenge, we used the model of subcutaneous immunization of C57BL/6 mice followed by an aerogenic challenge with virulent *M. tuberculosis* (McGuire et al., 2002). Groups of mice were vaccinated subcutaneously with either 1×10^6 BCG 9 or 1×10^6 *M. tuberculosis* $\Delta RD1$. Eight months following vaccination, the mice were all healthy, thereby demonstrating attenuation in a third mouse strain. Vaccinated and unvaccinated mice were aerogenically challenged with 200 CFU of the acriflavin-resistant strain of *M. tuberculosis*

Erdman. Twenty-eight days after the challenge, the mice were sacrificed and the bacterial burden in the lungs and spleens were determined (see Table 5). Naive mice served as controls. While the acriflavin-resistant *M. tuberculosis* grew to 6.61 ± 0.13 (\log^{10} CFU) in lungs of naive mice, both the BCG-vaccinated and *M. tuberculosis* $\Delta RD1$ -vaccinated mice exhibited greater than 1 log protection in lungs with CFU values of 5.07 ± 0.10 ($p < 0.001$ relative to controls) and 5.11 ± 0.14 ($p < 0.001$), respectively, detected at the four week time point. The *M. tuberculosis* $\Delta RD1$ also protected against hematogenous spread; CFU values in the spleen were 5.26 ± 0.11 for the controls, 4.00 ± 0.33 ($p < 0.01$) for the *M. tuberculosis* $\Delta RD1$ immunized mice, and 3.85 ± 0.17 ($p < 0.01$) for the BCG vaccinated animals. Thus, the *M. tuberculosis* $\Delta RD1$ shares long-term immunogenicity like BCG.

Table 5. Bacterial burden of virulent *M. tuberculosis* in uninoculated mice and mice inoculated with BCG and H37Rv $\Delta RD1$.

Vaccination strain	Lung (\log_{10} CFU)	Spleen (\log_{10} CFU)
-	6.61 ± 0.13	5.26 ± 0.11
BCG	$5.07 \pm 0.10^{***}$	$3.85 \pm 0.17^{**}$
H37Rv $\Delta RD1$	$5.11 \pm 0.14^{***}$	$4.00 \pm 0.33^{**}$

** $p < 0.01$; *** $p < 0.001$.

Discussion

BCG is a mutant of *M. bovis* that was isolated over 94 years ago and characterized for its attenuation for virulence in animals. For over 80 years, BCG has been used as a tuberculosis vaccine having been given to 3 billion humans. It is currently the only anti-tuberculous vaccine available for use in humans, yet its precise attenuating mutations and mechanisms of attenuation have never been determined. Previous studies had identified regions of the *M. bovis* chromosome that were absent from BCG, but present in virulent *M. bovis*

and *M. tuberculosis* strains (Mahairas et al., 1996; Gordon et al., 2001). An elegant microarray analysis has also demonstrated that there was only one deletion common to all BCG strains; the authors hypothesized this was the primary attenuating mutation in the original BCG strain isolated by Drs. Calmette and Guérin (Behr et al., 1999).

Using a combination of targeted deletion mutagenesis, virulence assays, and complementation analysis, we have been able to unambiguously prove that *RD1* is required for virulence for *M. tuberculosis*, and by analogy for *M. bovis*, for the first time.

Moreover, the combination of phenotypes associated with the early BCG strains: i) the attenuation for virulence, ii) the altered colonial morphotypes, and iii) the ability to confer long-term immunogenicity in animals allow us to conclude that the *RD1* deletion was the primary attenuating mutation in the original BCG isolate.

With regards to the $\Delta RD1$ mutant histology, at 22 weeks post infection, it was noted that the mutant was limited in the extent of the spread of infection, in contrast to the extensive damage caused by the parental strain. Interestingly, Pethe et al. (2001) determined that *M. tuberculosis* needs to bind and/or invade epithelial cells in order to disseminate and cause widespread destruction of the lung, whilst another study reported that pulmonary M cells can act as a portal of entry to the lung for the tubercle bacilli (Teitelbaum, 1999). In relation to *in vitro* analyses, studies utilizing a model of the alveolar barrier, consisting of pneumocytes and monocytes, described how *M. tuberculosis* infection of the pneumocytes resulted in cytolysis, which disrupted the barrier and allowed more efficient translocation of intracellular bacilli (Bermudez et al., 2002).

Notes

¹The following four primers were used to amplify upstream and downstream flanking sequences (UFS and DFS, respectively) for the construction of the *RD1* deletion mutants. UFS was amplified using TH201: GGGGGCGCACCTCAAACC and TH202: ATGTGCCAATCGTCGACCAGAA. DFS

²The unmarked deletion mutant of *M. tuberculosis* H37Rv, mc²4002, was generated by transformation using a *sacB* counterselection (Snapper et al., 1988; Pelicic et al., 1996; Pavelka et al., 1999). Specifically, the plasmid pJH508 was created by first cloning UFS into *KpnI* and *XbaI* sites, then cloning DFS into *EcoRI* and *HindIII* sites of pJH12, a pMV261-derived *E. coli* - *Mycobacteria* shuttle plasmid, to create pJH506 in which UFS and DFS flanked a green fluorescent protein gene (GFPuv, Clontech) whose expression was driven by the *M. leprae* 18Kd promoter. The UFS-gfp-DFS cassette was sub-cloned into the *EcoRV* site of plasmid pYUB657 to create pJH508. The first homologous recombination involved the identification of hygromycin resistant colonies, resulting from the transformation of *M. tuberculosis* with pJH508. Southern analysis of the *NcoI*-digested DNA isolated from hygromycin resistant colonies probed with UFS or DFS, confirmed the presence of a single copy of pJH508 inserted into the *M. tuberculosis* genome. The transformant (mc²4000) identified was then grown in 7H9 broth to saturation, to allow the second homologous recombination to occur, resulting in recombinants that could be selected by plating the culture on 7H10 plates, supplemented with 3% sucrose. Both Southern analysis and PCR of the DNA isolated from sucrose resistant colonies confirmed the RD1 deletion.

³Specialized transduction is a mycobacteriophage-based method for the delivery of homologous DNA constructs using conditionally replicating shuttle phasmids (Jacobs et al., 1987; Bardarov et al., 1997; Carriere et al., 1997) has been used successfully for *M. tuberculosis* (Glickman et al., 2000, 2001; Raman et al., 2001). Specifically, a transducing phage phAEKO1 was constructed by inserting UFS and DFS into pJSC347, flanking a hygromycin cassette, to create pJH313. pJH313 was digested with PacI and ligated to phAE159, a

temperature-sensitive mycobacteriophage derived from TM4. The transduction was performed by growing *M. tuberculosis* to an O.D.₆₀₀ of 1.0, washing twice with MP buffer (50 mM Tris pH 7.6, 150 mM NaCl, 10 mM MgCl₂, 2 mM CaCl₂), resuspending into an equal volume of MP buffer and mixing with the transducing phage phAEKO1 at an MOI of 10. The mixtures were incubated at 37°C overnight, then plated on 7H10 plates supplemented with hygromycin at 50 µg/ml. Hygromycin resistant colonies were analyzed by PCR and Southern analysis, as described above, to confirm the deletion of *RD1*.

⁴Complementation analyses was performed using the integration proficient cosmids (Skjot et al., 2000; van Pinxteren et al., 2000) pYUB412 made by S. Bardarov, a library made by F. Bange, and cosmid identified and generously provided by S.T. Cole.

Example 5. Vaccine efficacy of a lysine auxotroph of *M. tuberculosis*

In this Example, we describe the *in vivo* growth phenotype and vaccine efficacy of a lysine auxotrophic mutant of *Mycobacterium tuberculosis* strain H37Rv. An immunization experiment using the mouse model with an aerosol challenge showed that two doses of the *M. tuberculosis* mutant were required to generate protection equivalent to that of the BCG vaccine.

Despite the existence of anti-microbial drugs and a widely used vaccine, *Mycobacterium tuberculosis* remains the primary cause of adult death due to a bacterial agent (Dolin et al., 1994). The emergence of multi-drug resistant strains of *M. tuberculosis*, the variable efficacy of the current vaccine, the bacille-Calmette and Guérin (BCG), and the HIV pandemic have all contributed to a growing global tuberculosis problem.

Several studies have described the development of attenuated auxotrophic strains of BCG and/or *M. tuberculosis* (Guleria et al., 1996); Hondalus et al., 2000; Jackson et al., 1999; Smith et al., 2001). All of these studies utilized single immunization protocols and demonstrated differences in the protective responses thus elicited. In this study, we describe the *in vivo*

growth characteristics of a previously described lysine auxotroph of *M. tuberculosis* H37Rv (Pavelka and Jacobs, 1999), and evaluate the vaccine potential of this mutant by a multiple immunization protocol in a mouse model of the human disease, using an aerosol challenge.

Clearance of the *M. tuberculosis* lysine auxotroph in SCID mice. Female SCID mice were bred at the animal facility of the Albert Einstein College of Medicine. The animals were maintained under barrier conditions and fed sterilized commercial mouse chow and water ad libitum. The *M. tuberculosis* strains Erdman, mc²3026 (Δ lysA::res) (Id.), and mc²3026 bearing pYUB651 (expressing the wild-type *lysA* gene) were grown in Middlebrook 7H9 broth (Difco) supplemented with 0.05% Tween-80, 0.2% glycerol, 1X ADS (0.5% bovine serum albumin, fraction V (Roche); 0.2% dextrose; and 0.85% NaCl) or on Middlebrook 7H10 or 7H11 solid medium (Difco) supplemented with 0.2% glycerol and 10% OADC (Becton Dickinson). Culture media for the lysine auxotroph were supplemented with 1 mg/ml of L-lysine (for both liquid and solid media), and 0.05% Tween-80 was also added to solid medium. Liquid cultures were grown in 490 cm² roller bottles (Corning) at 4-6 rpm. Plates were incubated for 3-6 weeks in plate cans. All cultures were incubated at 37°C.

Titered frozen stocks of the bacteria were thawed and diluted appropriately in phosphate buffered saline containing 0.05% Tween-80 (PBST). The bacterial suspensions were plated at the time of injection to confirm the number of viable bacteria. Intravenous injections were given via a lateral tail vein. At various time points post-injection (24 hours, then once weekly), 3 mice were sacrificed, and the lungs, liver, and spleen removed and homogenized separately in PBST using a Stomacher 80 (Tekmar, Cincinnati, OH). The homogenates were diluted in PBST and plated to determine the number of CFU/organ. Note that mice were sacrificed at 24 hours post-injection in order to compare the bacterial colony forming units recovered from the mice with the colony forming units in the suspensions at the time of

injection. Thus the bacterial counts reported at time zero actually represent the viable bacteria recovered from the mice at 24-hours post-injection.

The lysine auxotrophic strain was cleared from and did not appear to grow in the examined organs of the SCID mice, while the complemented strain multiplied extensively (FIG. 14). Interestingly, the auxotrophic inoculum was cleared from the spleens and lungs but persisted somewhat longer in the liver (FIG. 14B). The mice receiving the complemented *M. tuberculosis* mutant died within three weeks of challenge, while the mice given the auxotrophic *M. tuberculosis* mutant did not display any gross organ pathology and survived for at least the duration of the experiment.

Two immunizations with the *M. tuberculosis* lysine auxotroph mc²3026 are required to match the efficacy of vaccination with BCG-Pasteur. We tested the vaccine potential of the lysine auxotroph mc²3026 in the mouse model by means of a virulent aerosol challenge. Female, pathogen-free C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were vaccinated intravenously with ca. 1×10^6 CFU of the *M. tuberculosis* lysine auxotroph or BCG-Pasteur suspended in 0.2 ml PBST. Mice vaccinated with mc²3026 were revaccinated at 4 week intervals and the number of viable organisms in the lungs and spleens determined weekly throughout the vaccination period, as described above for the SCID mouse experiments. Five mice were examined at each time point.

Immunized mice were challenged 3 months after the initial vaccination. A frozen aliquot of a *M. tuberculosis* Erdman stock was thawed and diluted in PBST to ca. 1×10^6 CFU/ml and 10 ml was introduced into the nebulizer of a Middlebrook aerosol chamber (Glas-Col, Terre Haute, IN). The mice were exposed to the infectious aerosol for 30 minutes, inhaling 50-100 CFU into their lungs over this period. Five mice were sacrificed immediately following the challenge period and the lung homogenates were plated to check the amount of the challenge inoculum actually reaching the lungs. Groups of vaccinated and control mice were sacrificed 14, 28, and 42 days later and the lung and spleen homogenates plated to determine the number of viable colony forming units of

M. tuberculosis Erdman present. Data were analyzed using the Student's t-test and an analysis of variance between several independent means, using the In Stat Statistics program (GraphPad Software, San Diego).

A preliminary experiment demonstrated that a single intravenous immunization of immunocompetent C57BL/6 mice with the *M. tuberculosis* mutant did not generate a significant protective response to the subsequent aerosol challenge with virulent *M. tuberculosis* Erdman. In that experiment, the *M. tuberculosis* auxotroph was rapidly cleared from the mice (FIG. 15A), and the single immunization with the auxotroph was insufficient to reduce the bacterial burden in the lungs and spleens relative to a single immunization with BCG (FIG. 15B).

The failure of the auxotroph to confer protection might have been due to the inability of the mutant to persist long enough, or to synthesize enough antigen to induce an immune response that could significantly restrict the growth of the challenge organisms. One way to circumvent this problem is to give multiple doses of vaccine (Collins, 1991; Homchampa et al., 1992). To this end, mice were intravenously immunized two or three times at four-week intervals with the *M. tuberculosis* lysine auxotroph. In both cases, the vaccine strain was cleared from the lungs and spleens of all the mice at rates similar to that seen with the single immunization experiment (FIG. 15A). Three months after the first immunization the mice were challenged with *M. tuberculosis* Erdman by the aerosol route and the bacterial counts in the lungs and spleens were determined and compared to a BCG-Pasteur immunized control, as well as the sham immunized controls. As seen in FIG. 15C, double immunization with the *M. tuberculosis* lysine auxotroph induced a protective response that was equivalent to that of the BCG control. The reduction in counts in the lung and spleen was equivalent to a 100-fold reduction in bacterial counts compared to the unvaccinated control (FIG. 15C). The results from the triple immunization experiment were essentially similar as those from the double immunization experiment described above (data not shown). Furthermore, mice that were

immunized with three doses of the *M. tuberculosis* lysine auxotroph and challenged with virulent *M. tuberculosis* Erdman survived at least as long as the BCG-immunized control mice (FIG. 16).

Several studies have described the development and vaccine efficacy of attenuated mutant strains of *M. tuberculosis* (Jackson et al., 1999; Hondalus et al., 2000; Smith et al., 2001). The first study reported that a purine auxotroph of *M. tuberculosis* was unable to grow in macrophages and was attenuated for growth in both mice and guinea pigs (Jackson et al., 1999). A guinea pig vaccination experiment determined that a single immunization with the auxotroph allowed the animals to restrict the growth of virulent *M. tuberculosis* in the lungs as well as a single immunization with wild-type BCG, following aerosol challenge. However, the reduction in growth of the challenge organism in the spleen afforded by the auxotroph was not as extensive as that afforded by BCG. Another study reported that a leucine auxotroph of *M. tuberculosis* Erdman cannot grow in macrophages and is avirulent to immunocompromised SCID mice (Hondalus et al., 2000). Immunocompetent mice vaccinated once with a *M. tuberculosis* leucine mutant did not significantly restrict the growth of the virulent challenge organism in the lungs or spleen as much as the control mice vaccinated with BCG (Id.). However, the mice immunized with the leucine auxotroph survived as long as the BCG immunized controls and exhibited a decreased histopathology relative to that seen in the non-immunized controls (Id.). A third study showed that *M. tuberculosis* proline and tryptophan auxotrophs were attenuated and a single immunization of mice with either of these mutants afforded protection against an intravenous challenge with virulent *M. tuberculosis*, comparable to that for BCG, as indicated by the mean survival times (Smith et al., 2001). In those experiments, mice immunized with *pro* or *trp* mutants could restrict the growth of the challenge organisms to the same extent as mice immunized with BCG, although the magnitude of protection in either case (*M. tuberculosis* auxotrophs or BCG) was not as extensive as that seen in the other studies (Id.).

In the present study we have demonstrated that a single immunization of mice with the avirulent *M. tuberculosis* lysine auxotroph did not generate an immune response capable of significantly restricting the growth of virulent *M. tuberculosis* Erdman following an aerogenic challenge. However, administration of a second or a third dose of this vaccine increased protection substantially, as measured by the number of viable bacteria per organ, to a level similar to that achieved with single dose of BCG-Pasteur. This level of protection did not seem to be greatly increased by a third dose of vaccine, although the triply immunized mice survived as long as the control mice immunized with a single dose of BCG-Pasteur. Mice that were immunized twice were not followed to determine mean survival time, but comparing the growth curves of the challenge bacteria following the double and triple immunizations, it seems likely that the survival time for the doubly immunized mice would be much the same as that for the triple-immunized mice.

The previous studies using *M. tuberculosis* auxotrophs as vaccine strains showed substantial variations in their effectiveness. This variability is likely to be due to a number of factors, including the different *M. tuberculosis* background strains used to construct the mutants, different mouse strains used in the various protection studies, and the different challenge organisms and challenge routes used. There was also considerable variation in the protective efficacy of the different vaccines compared to that observed in controls using BCG immunization. These differences pose a number of questions concerning the best indicators of protection, especially in the long term. Should viable bacterial counts or survival be the primary indicator of protection or should both be given equal weight? The results of this study indicate that more than one immunization with a *M. tuberculosis* lysine auxotroph did generate a significant protective response as indicated by both criteria. We believe it is important that multiple immunization protocols be considered in the further development of attenuated *M. tuberculosis* strains as potential human vaccines.

This is the first study demonstrating that a multiple immunization protocol using an auxotroph of *M. tuberculosis* can protect against a highly virulent aerosol challenge compared to that seen for BCG. Since BCG vaccines have shown variable efficacy when tested in humans, an auxotrophic *M. tuberculosis* vaccine might represent an attractive booster vaccine with which to augment childhood BCG immunization.

Example 6. Mutants of *Mycobacterium tuberculosis* having two attenuating mutations are safe and provide protection in mammals lacking CD4⁺ lymphocytes.

The experiments described in this Example employ materials and methods described in the other Examples.

Construction and characterization of *M. tuberculosis* $\Delta RD1 \Delta panCD$ (mc²6030).

A pantothenate auxotroph of *M. tuberculosis* $\Delta RD1$ was generated by specialized transduction and the strain designated mc²6030. No CFU were detected on 7H11 when 5×10^{10} CFU were plated (repeated twice), suggesting the reversion frequency to be below 10^{-11} .

SCID mice infected with 1×10^2 CFU H37Rv succumbed to infection in 6 weeks, whereas the mice infected with 1×10^6 mc²6030 survived significantly longer with more than 75% of mice surviving for more than 300 days (FIG. 17A). Bacteria isolated from mc²6030-infected mice before they died were all auxotrophs, confirming that there were no revertants under in vivo conditions. In order to assess the safety of mc²6030 in immunocompetent BALB/c mice, we infected mice intravenously with 1×10^6 mc²6030 or 1×10^6 of wild-type H37Rv. All mice infected with H37Rv succumbed to infection by 150 days, whereas mice infected with mc²6030 survived for more than 300 days (FIG. 17B). In an effort to understand the role of immune responses in controlling infection with the pantothenate mutants, we infected immunocompetent C57Bl/6 with 1×10^6 CFU of mc²6001 ($\Delta RD1$), mc²6004 (complementing strain), mc²6030 ($\Delta RD1 \Delta panCD$) or wild-type H37Rv. Mice infected with H37Rv and mc²6004

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showed progressive growth in all the three organs, whereas mice infected with mc²6030 showed a drop in growth during the first 3 weeks in the lungs and spleen (FIG. 18). Following 3 weeks of infection, the growth pattern of both mc²6001 and mc²6030 were identical in the spleen and lungs. Mice immunized subcutaneously with one or two doses of mc²6030 demonstrated protection against aerosol challenge with virulent *M. tuberculosis*, which was comparable to the protection afforded by BCG vaccination (Table 6). No pantothenate auxotrophs were recovered from spleen or lungs of mice at 1, 2 or 3 months following subcutaneous immunization.

Table 6. Bacterial burden of virulent *M. tuberculosis* in uninoculated mice and mice inoculated with BCG or one or two doses of $\Delta RD1\Delta panCD$.

Experimental Group	Lung CFUs (\log_{10})	Spleen CFUs (\log_{10})
Naive	5.99 ± 0.09	4.94 ± 0.06
$\Delta RD1\Delta panCD$ (1 dose) sc	$5.22 \pm 0.10^*$	$4.04 \pm 0.15^*$
$\Delta RD1\Delta panCD$ (2 doses) sc	$4.86 \pm 0.14^{**}$	$3.58 \pm 0.11^{**}$
BCG (1 dose) sc	$4.79 \pm 0.19^{**}$	$3.73 \pm 0.27^{**}$

* $p < 0.01$ relative to controls; ** $p < 0.001$ relative to controls

Construction and characterization of *M. tuberculosis* $\Delta lysA\Delta panCD$ (mc²6020).

A pantothenate auxotroph of *M. tuberculosis* $\Delta lysA$ was generated by specialized transduction and the strain designated mc²6020. No CFU were detected on 7H11 when 5×10^{10} CFU were plated, suggesting the reversion frequency to be below 10^{-11} . This double mutant is auxotrophic for both lysine and pantothenate. SCID mice infected with 1×10^2 CFU H37Rv succumbed to infection in 6 weeks, whereas the mice infected with 1×10^6 mc²6020 survived for more than 400 days with no mortality. In order to assess the safety and growth kinetics of mc²6020 in immunocompetent BALB/c mice, we infected mice intravenously with 1×10^6 mc²6020 or 1×10^6 of wild-type H37Rv. All mice infected with H37Rv succumbed to infection by 150 days, whereas mice infected with mc²6020 survived for more than 400 days. After 3 weeks following intravenous infection, no colonies of mc²6020 could be recovered from spleen, liver or lungs of infected mice. Interestingly, mice immunized subcutaneously with one or two doses of mc²6020 demonstrated protection against aerosol challenge with virulent *M. tuberculosis*, which was comparable to the protection afforded by BCG vaccination (Table 7). No pantothenate and lysine requiring auxotrophs were recovered from spleen or lungs of mice at 1, 2 or 3 months following subcutaneous immunization. Other studies established

that both mc²6020 and mc²6030 protects the a level of protection of mice against TB equivalent to the protection afforded by BCG (FIG. 19).

Table 7. Bacterial burden of virulent *M. tuberculosis* in uninoculated mice and mice inoculated with BCG or one or two doses of mc²6020 (Δ lysA Δ panCD) sc or one dose of mc²6020 iv.

Experimental group	Lung CFUs (log ₁₀)	Spleen CFUs (log ₁₀)
naive	6.03 \pm 0.05 ^a	4.84 \pm 0.27
BCG (1 dose) sc	4.76 \pm 0.19***	3.95 \pm 0.18*
mc ² 6020 (1 dose) sc	5.05 \pm 0.06***	4.02 \pm 0.11*
mc ² 6020 (2 doses) sc	5.09 \pm 0.05***	4.06 \pm 0.27
mc ² 6020 (1 dose) iv	5.06 \pm 0.11***	4.00 \pm 0.15*

^aMean \pm SEM p<0.001 = ***; p<0.05 = *

These data clearly demonstrate the safety and immunogenicity of these two double mutants of *M. tuberculosis* in mice.

The double deletion mutant mc²6030 (Δ RD1 Δ panCD) immunizes and protects CD4^{-/-} mice from aerosolized *M. tuberculosis* challenge. We tested the

hypothesis that the attenuated double deletion mutants could protect CD4-deficient mice, a model of HIV-infected humans, from aerosolized *M. tuberculosis* challenge. The results of these tests are summarized in FIG. 20.

While 100% of the non-immunized CD4^{-/-} were dead by 38 days, 100% of the mice immunized with either BCG or mc²6030 are alive at 120 days post challenge. After 120 days, none of the immunized mice had any outward sign of disease. The double deletion mutants were also safer than BCG in SCID mice, where all of the SCID mice died before 100 days when inoculated with BCG, 100% and 25% of the mice survived inoculation with mc²6020 and mc²6030, respectively (FIG. 21). This indicates that immunity against *M. tuberculosis* can be elicited in a CD4-independent manner. These results also

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support the notion that effective antibody or CD8-mediated vaccines to malaria and HIV could be developed in the context of these attenuated *M. tuberculosis* strains.

In view of the above, it will be seen that the several advantages of the invention are achieved and other advantages attained.

As various changes could be made in the above methods and compositions without departing from the scope of the invention, it is intended that all matter contained in the above description and shown in the accompanying drawings shall be interpreted as illustrative and not in a limiting sense.

All references cited in this specification are hereby incorporated by reference. The discussion of the references herein is intended merely to summarize the assertions made by the authors and no admission is made that any reference constitutes prior art. Applicants reserve the right to challenge the accuracy and pertinence of the cited references.

SEQ ID Nos

SEQ ID NO:1 - An RD1 region of *Mycobacterium tuberculosis* H37Rv.

Bases 4350263-4359716 of the genome of *M. tuberculosis* H37Rv, as provided in GenBank Accession No. NC000962.

4350263 gatcgtgg gtgcgcgcgg ggggatgccg ccgatggcac
4350301 cgctggcccc gttattgccg gcggcggcag atatcgggtt gcacatcatt gtcacctgtc
4350361 agatgagcca ggcttacaag gcaaccatgg acaagtctgt cggcgccgca ttcgggtcgg
4350421 gcgctccgac aatgttccct tcgggcgaga agcaggaatt cccatccagt gaggttcaagg
4350481 tcaagcggcg cccccctggc caggcatttc tcgtctcgcc agacggcaaa gaggtcatcc
4350541 agggccccct catcgagcct ccagaagaag tgttcgcagc accccaagc gccggttaag
4350601 attatttcat tgccggtgta gcaggaccgc agctcagccc ggtaatcgag ttcgggcaat
4350661 gctgaccatc ggggttgttt ccggctataa ccgaacgggt tgtgtacggg atacaaatac
4350721 agggagggaa gaagtaggca aatggaaaaa atgtcacatg atccgatcgc tgcgcacatt
4350781 ggcacgcaag tgagcgacaa cgctctgcac ggctgacgg cgggctcgac ggcgctgacg
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4351021 gccggcgtct tcgcgaata ggcccccaac acatcggagg gagtgatcac catgctgtgg
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4351441 ttcctcggta tcaacacgat ccgatcgcg ttgaccgaga tggattattt catccgtatg
4351501 tggaaaccagg cagccctggc aatggaggtc taccaggccg agaccgcggt taacacgctt
4351561 ttcgagaagc tcgagccgat ggctcgatc cttgatcccg gcgcgagcca gagcacgacg
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4352101 ctggtcgcgc cggcaccgct cgcgcaggag cgtgaagaag acgacgagga cgaactgggac
4352161 gaagaggacg actggtgagc tcccgtaatg acaacagact tcccgccac ccgggcccga
4352221 agacttgcca acattttggc gaggaaggta aagagagaaa gtagtccagc atggcagaga
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4352341 acctgaaaac ccagatcgac caggtggagt cgacggcagg ttctgtgcag ggccagtggc
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4352461 agcagaagca ggaactcgac gagatctcga cgaatattcg tcaggccggc gtccaatact
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4353001 gttgtcatgg cggccgacta cgacaagctc ttccggcgcc acgaaggat ggaagctccg
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4353301 acacccccca tgcccatcgc cggaccgga cggcccccac ccaaaccacc cacaccccc
4353361 atgcccacgc ccggaccgga accggcccc cccaaaccac ccacacctcc gatgcccatc
4353421 gccggacctg cccccacccc aaccgaatcc cagttggcgc ccccagacc accgacacca
4353481 caaacgccaa ccggagcgcc gcagcaaccg gaatcaccgg cggccacgt accctcgac
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 4356001 cggaggctac cagcgaagaa accccgacct ggcaggccat catcgcgctg gtgcccgcgt
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 4356481 tccccatgct gctgtggatc accggggtgt acgacacggg ccgcaatatc cgtttctgag
 4356541 ccggatcggc tgattggcgg ttctgacag aacatcgagg acacggcgca ggtttgcata
 4356601 ccttcggcgcc ccgacaaatt gctgcgattg agcgtgtggc gcgtccggta aaatttgctc
 4356661 gatgggggaa acgtatagga gatccggcaa tggtggaacc gttggcgctc gatcccaccg
 4356721 gcttgagcgc agcggccgcg aaattggccg gcctcgtttt tccgcagcct ccggcgccga
 4356781 tcgcgggtcag cggaaaggat tcggtggtag cagcaatcaa cgagaccatg ccaagcatcg
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 4356901 acatgaacgc ggcggcggaac gtctatgcga agaccgatca gtcactggga accagtttga
 4356961 gccagtatgc attcggctcg tcgggcgaag gcctggctgg cgtcgctcg gtcgggtggtc
 4357021 agccaagtca ggctaccag ctgctgagca caccgtgtc acaggtcacg accagctcg
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 4357561 acgcagcgcc tgttgctgtc ctggcctcgt cagcatgcgg cggccagggc ccggtcgagc
 4357621 aaccgggtga ogtattgcca gtacagccag tccgcgacgg ccacacgctg gacggccgcg
 4357681 tcagtcgcag tgtgcgcttg gtgcagggca atctcctgtg agtgggcagc gtaggcccg

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 4359481 ccggcaatat gcctgtgcca ggtaatcacc gtggcgagat aatcctgcag cgtcatcaat
 4359541 tgattgatgt ttgcaccag ggcgcggtt gcagcattgg cggcgccgcg ggaccatagg
 4359601 ccgccttcga agacgtggcc tttctgctgg cggcaggtgt ccaatacatc ggtgacctt
 4359661 tgcaaaacct ggtatatctc ctgggcccgc tcatagaaag tgtcttcacg ggcttc

SEQ ID NO:2 - A *panCD* region of *Mycobacterium tuberculosis* H37Rv.

Deleted in Δ *panCD* strain of Example 2.

GGTCTAGCAGCTCGCCCGCGTTTTCGGGCACAAATGCCGGATCGTGGCCCATGTCGATCGGTTTGTGTGTA
AGCGTCGACAAACACGATCCCGCGGCTGGTATGTGCGGGCCCGGGCGTCTGTCATCGTCGCGTACGCAATC
AGAATCACCAGATCCCCCGGATGCACCAAGTGC CGCGGGCACC GTTGATGCCAATCACACCACTGCCGC
GTTCCGCCGGTGATCGCGTAGGTGACCAAGTCGAGCACC GTTGTGATATCGACGATGGTTACCTGTTCCGC
TTCCAGCAGGTCCGCGCGGTCCATCAAGTCGGCATCGATGGTCACCGAGCCGACGTAGTGCAGGTCCGGCG
CAGGTACCCGTGGCGCGGTGGATCTTCGACTTCAGCATCGTCCGTAAACATCAGTTTCTCCAATGTGATTC
GAGGATTGCCCCGGTATCCGTCCGGGCGGTTCGGTCCCGGCGAAAGTTCCGATTTCAATCGCAATGTTGTCC
AGCAGCCTGGTGGTGCCAAGCCGGGCGAGCAACCAGCAGCCGACCGGAACCGTTGAGCGGCATCGGGCCAA
GCCCCGATATCGCGCAGCTCCAGGTAGTCGACCGCCACGCCGGGTGCGAGCTCGAGCACCACGGGCGGC
ATCCAGCGCGGCTGCGCGCCAGCCGTTGCCGCATGCGCTGCGGCCGTTAGCGCCGCGAGAGCGCGACG
GCCGCCGACGCTGGGCGGGTCCAGGTAGCGGTTGCGCGACGACATCGCCAGCCGTCGGCTTCGCGCA
CGGTCCGCGACGCCGACCACCGCGACATCGAGGTTGAAGTCCGCGACCAGCTGCCGGATCAGCACCAGCTG
CTGGTAGTCCTTCTCACCGAAGAACACCCGATCCGGGCGCAGCATCTGCAGCAGCTTTAGCAGCACCCTC
AGCAGCCCGCGAAATGGGTTGGCCGCGGGCCGCCCTCGAGTTCGGCGGCCAACCGGACCGGGTTGCACGG
TGGTGCAGCGCCGTCGGGATACATCGCCGCGGTAGTTGGCGTGAAAGCGATTTCCACGCCCTTCGGGCCG
CAGTTGCGCCAGGTCTGTCGTCGGGGTGC GGCGATAGGCGTCGAGATCTTCCCCGGCACC GAATTGCATC
GGGTTGACGAAGATCGACACGACGACGACCGATCCGGGCACCCGCTTGGCCGACGCAACGCGAGGT
GGCCTTCGTGCAGCGCACCCATAGTAGGCACCAACATCACTCGCCGGCCGGTGAGTCGAGTGCAGTGCAGT
GACATCGGCGACATCCCCCGGTGCCGAGTACACATTGA

SEQ ID NO:3 - A *nadBC* region of *Mycobacterium tuberculosis* H37Rv.

Deleted in Δ *nadBC* strain of Example 2.

AACGGGCGATGAGCCGGGACGCGTCGATGTACCGCGCCGCCCGGGCTGCACCGGCTGTGCGACAGCCT
ATCCGGAGCACAGGTTGCGCGACGTGGCTTGTGCGCCGCGATTTTCAGGACGTGGCGCTCACGCTGGTCGCG
CAGAGCGTGACCGCCGCCCTTGGCCCCGACCGAAAGCCGTGGCTGCCATCATCGCGCGGAGTACCCGT
GCACCGTGCCGGAGCAGGCACGCAGCATCGTGGTCCGGGGAGCCGACGACGCAATGCGGTGTGTGTCCA
GGCGCTAGTGGCGGTGTGCTGATGGGGTTATCCGACTGGGAGCTGGCTGCGGCTCGAGCAGCAATCGCGC
GTGGGCTCGACGAGGACCTCCGGTACGGCCCCGGATGTCAACACATTGGCGACGGTGCCCTGCCAGTGCAGC
GACCACCGCATCGCTGGTGACCCGGGAGGCCGGTGTGGTTGCCGATTGGATGTGCGCGCTGCTGACGCTG
AACGAAGTCCTGGGCACCAACGGTTATCGGGTGCTCGACCGCTCGAGGACGGCGCCCGGGTGCCCGCG
GAGAGGCACTTATGACGCTGGAAGCCCAAACGCGCGGATTGTTGACCGCCGAGCGCACCATGTTGAACCT
GGTCGGTCACTGTGCGGAATCGCCACCGCGACGGCCGCGTGGGTCGATGCTGTGCGCGGGACCAAGCG
AAAATCCGCGATACCCGTAAGACGCTGCCCCGCCCTGCGCGCGCTGCAAAAATACGCGGTGCGTACCGGTG
G

SEQ ID NO:4 *lysA* sequence in Δ *lysA* strains

GTGAACGAGCTGCTGCACTTAGCGCCGAATGTGTGGCCGCGCAATACTACTCGC
GATGAAGTCGGTGTGGTCTGCATCGCAGGAATTCCACTGACGCAGCTCGCCCAG
GAGTACGGGACCCCGCTGTTTCGTCATCGACGAGGACGACTTTCGCTCGCGCTGC
CGAGAAACCGCCGCGGCCTTTGGAAGTGGGGCGAACGTGCACTATGCCGCCAA
GGCGTTCCTGTGCAGCGAAGTAGCCCGGTGGATCAGCGAAGAAGGGCTCTGTC
TGGACGTTTGCACCGGTGGGGAGTTGGCGGTCGCGCTGCACGCTAGCTTTCGG
CCCGAGCGAATTACCTTGCACGGCAACAACAAATCGGTCTCAGAGTTGACCGCT
GCGGTCAAAGCCGGAGTCGGCCATATTGTCGTCGATTTCGATGACCGAGATCGA
GCGCCTCGACGCCATCGCGGGCGAGGCCGGAATCGTCCAGGATGTCCTGGTGC
GTCTCACCGTCGGTGTGCGAGGCGCACACCCACGAGTTCATCTCCACCGCGCACG
AGACGCGTCAGCCACATCGGTTTCGCAGATCTTCGACGTGGACGGCTTCGAACTC
GCCGCGCACCGTGTTCATCGGCCTGCTACGCGACGTCGTCGGCGAGTTCGGTCC
CGAAAAGACGGCACAGATCGCGACCGTCGATCTCGGTGGCGGCTTGGGCATCT
CGTATTTGCCGTCCGACGACCCACCGCCGATAGCCGAGCTCGCGGCCAAGCTG
GGTACCATCGTGAGCGACGAGTCAACGGCCGTGGGGCTGCCGACGCCCAAGCT
CGTTGTGGAGCCCGGACGCGCCATCGCCGGACCGGGCACCATCACGTTGTATG
AGGTCGGCACCGTTAAGGACGTCGATGTCAGCGCCACAGCGCATCGACGTTAC
GTCAGTGTCGACGGCGGCATGAGCGACAACATCCGCACCGCGCTCTACGGCGC
GCAGTATGACGTCCGGCTGGTGTCTCGAGTCAGCGACGCCCCGCCGGTACCGG
CCCGTCTGGTCGGAAAGCACTGCGAAAGTGGCGATATCATCGTGCGGGACACC
TGGGTGCCCCGACGATATTCGGCCCCGGCGATCTGGTTGCGGTTGCCGCCACCGG
CGCTTACTGCTATTCGCTGTGAGTCGTTACAACATGGTCGGCCGTCCCGCTGT
GGTAGCGGTGCACGCGGGCAACGCTCGCCTGGTCCTGCGTCGGGAGACGGTCG
ACGATTTGCTGAGTTTGGAAGTGAGGTGA

What is claimed is:

1. A method of treating a mammal that is deficient in CD4⁺ lymphocytes, the method comprising inoculating the mammal with an attenuated mycobacterium in the *Mycobacterium tuberculosis* (*M. tuberculosis*) complex, the mycobacterium comprising two deletions, wherein a virulent mycobacterium in the *M. tuberculosis* complex having either deletion exhibits attenuated virulence.
2. The method of claim 1, wherein the mammal is at risk for infection by a virulent mycobacterium in the *M. tuberculosis* complex.
3. The method of claim 1, wherein the attenuated mycobacterium is an *M. tuberculosis*.
4. The method of claim 3, wherein the *M. tuberculosis* is an H37Rv strain.
5. The method of claim 3, wherein the *M. tuberculosis* is a CDC1551 strain.
6. The method of claim 1, wherein the attenuated mycobacterium is an *M. bovis*.
7. The method of claim 1, wherein the mammal is a cow.
8. The method of claim 1, wherein the mammal is a human.
9. The method of claim 1, wherein the mammal is a human child.

10. The method of any one of claims 1-9, wherein at least one of the two deletions is a deletion of a region selected from the group consisting of an *RD1* region, a region controlling production of a vitamin, and a region controlling production of an amino acid.

11. The method of claim 10, wherein the deletion is of the *RD1* region.

12. The method of claim 11, wherein the deleted *RD1* region has at least 95% homology to SEQ ID NO:1.

13. The method of claim 11, wherein the deleted *RD1* region comprises SEQ ID NO:1.

14. The method of claim 10, wherein the deletion is of a region controlling production of a vitamin.

15. The method of claim 14, wherein the vitamin is pantothenic acid or nicotinamide adenine dinucleotide (NAD).

16. The method of claim 17, wherein the vitamin is pantothenic acid.

17. The method of claim 16, wherein the deletion is a Δ *panCD* deletion.

18. The method of claim 17, wherein the Δ *panCD* deletion has at least 95% homology to SEQ ID NO:2.

19. The method of claim 17, wherein the Δ *panCD* deletion comprises SEQ ID NO:2.

20. The method of claim 10, wherein the deletion is in a region controlling production of an amino acid.
21. The method of claim 20, wherein the amino acid is selected from the group consisting of proline, tryptophan, leucine or lysine.
22. The method of claim 20, wherein the amino acid is lysine.
23. The method of claim 22, wherein the deletion is a Δ lysA deletion.
24. The method of claim 23, wherein the Δ lysA deletion has at least 95% homology to SEQ ID NO:4.
25. The method of claim 23, wherein the Δ lysA deletion comprises SEQ ID NO:4.
26. The method of claim 10, wherein one deletion is of an *RD1* region and the other deletion is of a region that controls production of a vitamin.
27. The method of claim 26, wherein the deletion of the *RD1* region comprises SEQ ID NO:1 and the deletion of a region that controls production of a vitamin comprises SEQ ID NO:2.
28. The method of claim 10, wherein one deletion is of an *RD1* region and the other deletion is of a region that controls production of an amino acid.
29. The method of claim 28, wherein the deletion of the *RD1* region comprises SEQ ID NO:1 and the deletion of a region that controls production of an amino acid comprises SEQ ID NO:4.

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30. The method of claim 10, wherein one deletion is of a region that controls production of a vitamin and the other deletion is of a region that controls production of an amino acid.

31. The method of claim 30, wherein the deletion of a region that controls production of a vitamin comprises SEQ ID NO:2 and the deletion of a region that controls production of an amino acid comprises SEQ ID NO:4.

32. The method of any one of claims 1-32, wherein at least one of the deletions is made by genetic engineering.

33. The method of any one of claims 1-32, wherein the deletion is made by specialized transduction.

34. The method of any one of claims 1-32, wherein the deletion is made by sequential two-step recombination.

35. The method of claim 34, wherein the sequential two-step recombination uses a *sacB* selective marker.

36. The method of any one of claims 1-35, wherein the attenuated mycobacterium further comprises a foreign DNA stably integrated into genomic DNA of the mycobacterium.

37. The method of claim 36, wherein the foreign DNA encodes at least one protein or polypeptide selected from the group consisting of an antigen, an enzyme, a lymphokine, an immunopotentiator, and a reporter molecule.

38. The method of claim 37, wherein the foreign DNA encodes at least one protein antigen selected from the group consisting of antigens from

Mycobacterium leprae, *Mycobacterium tuberculosis*, malaria sporozoites, malaria merozoites, diphtheria toxoid, tetanus toxoids, *Leishmania spp.*, *Salmonella spp.*, *Mycobacterium africanum*, *Mycobacterium intracellulare*, *Mycobacterium avium*, *Treponema spp.*, Pertussis, Herpes virus, Measles virus, Mumps virus, *Shigella spp.*, *Neisseria spp.*, *Borrelia spp.*, rabies, polio virus, Human immunodeficiency virus, snake venom, insect venom, and *Vibrio cholera*; steroid enzymes; interleukins 1 through 7; tumor necrosis factor α and β ; interferon α , β , and γ ; and reporter molecules luciferase, β -galactosidase, β -glucuronidase and catechol dehydrogenase.

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Abstract

Methods of treating a mammal that is deficient in CD4⁺ lymphocytes are provided. The methods comprise inoculating the mammal with an attenuated mycobacterium in the *M. tuberculosis* complex. In these methods, the mycobacterium comprises two deletions, wherein a virulent mycobacterium in the *M. tuberculosis* complex having either deletion exhibits attenuated virulence.

Fig. 1

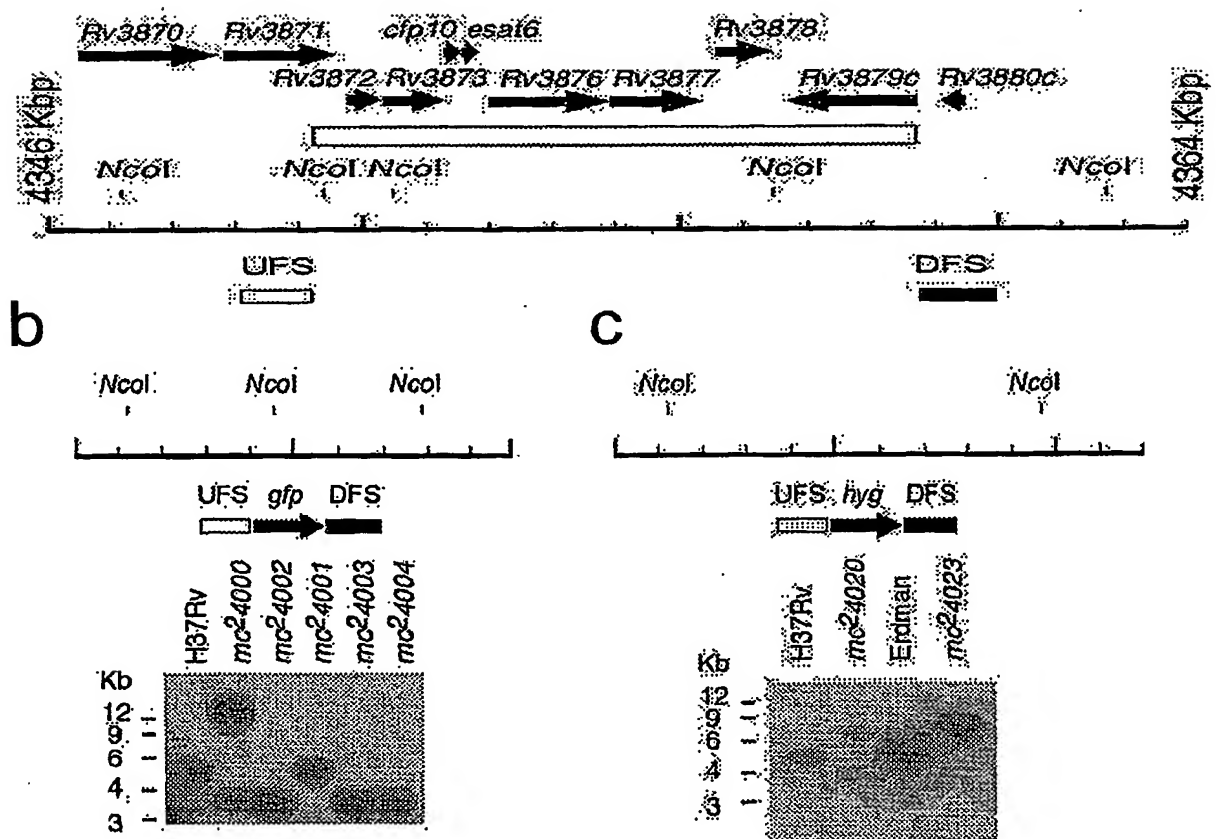


Fig. 2

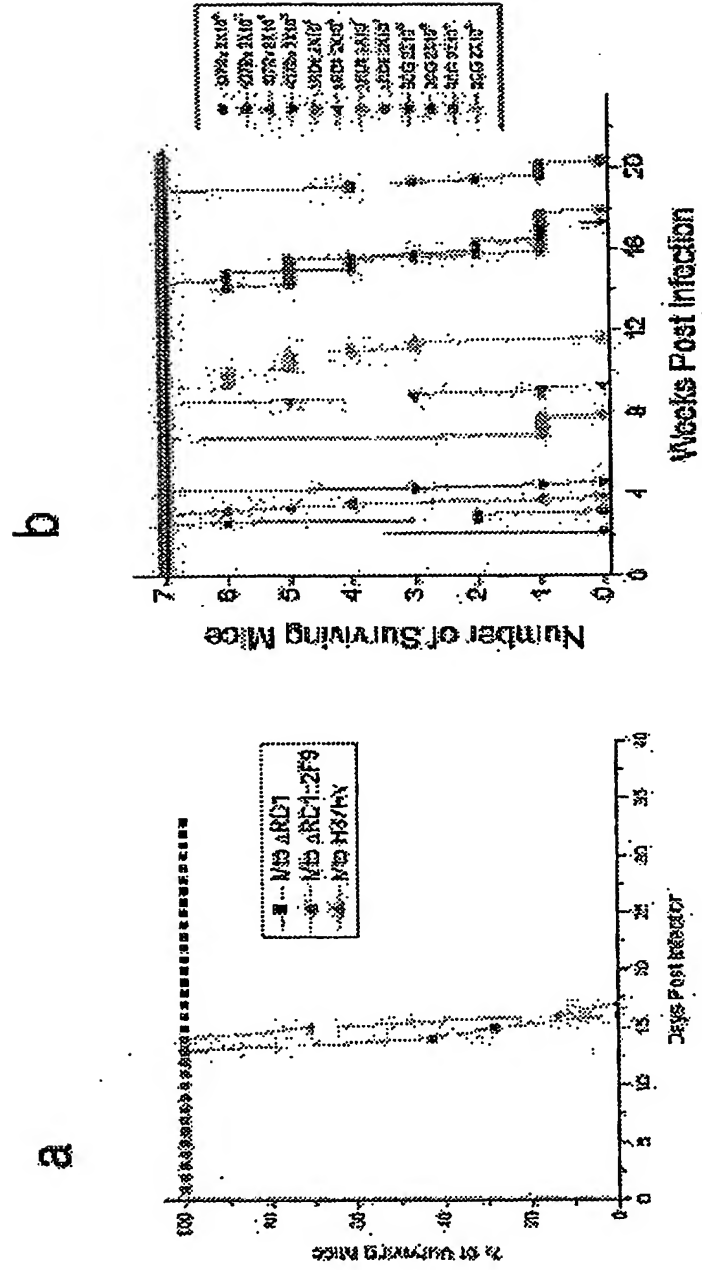


Fig. 3

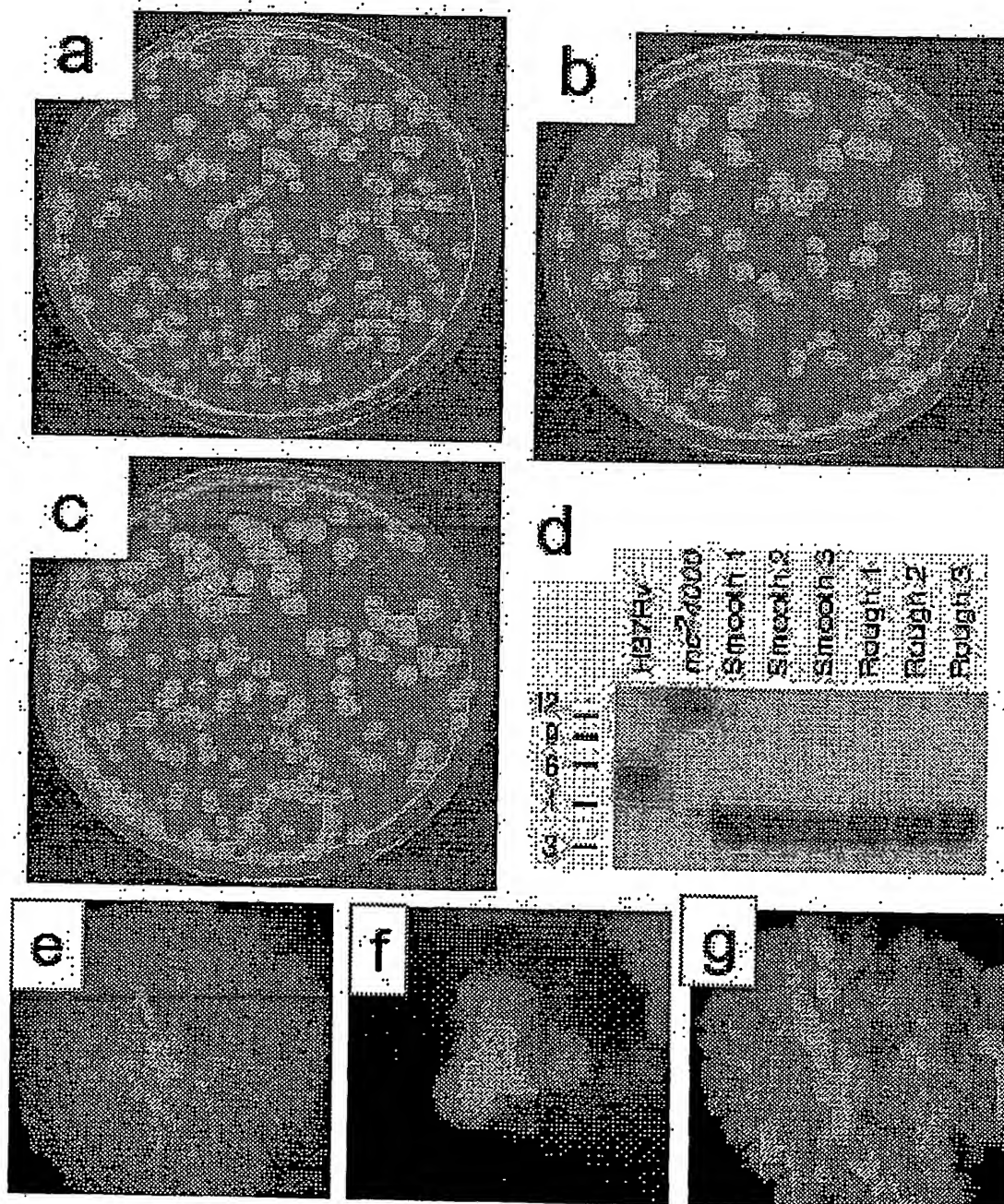


Fig. 4

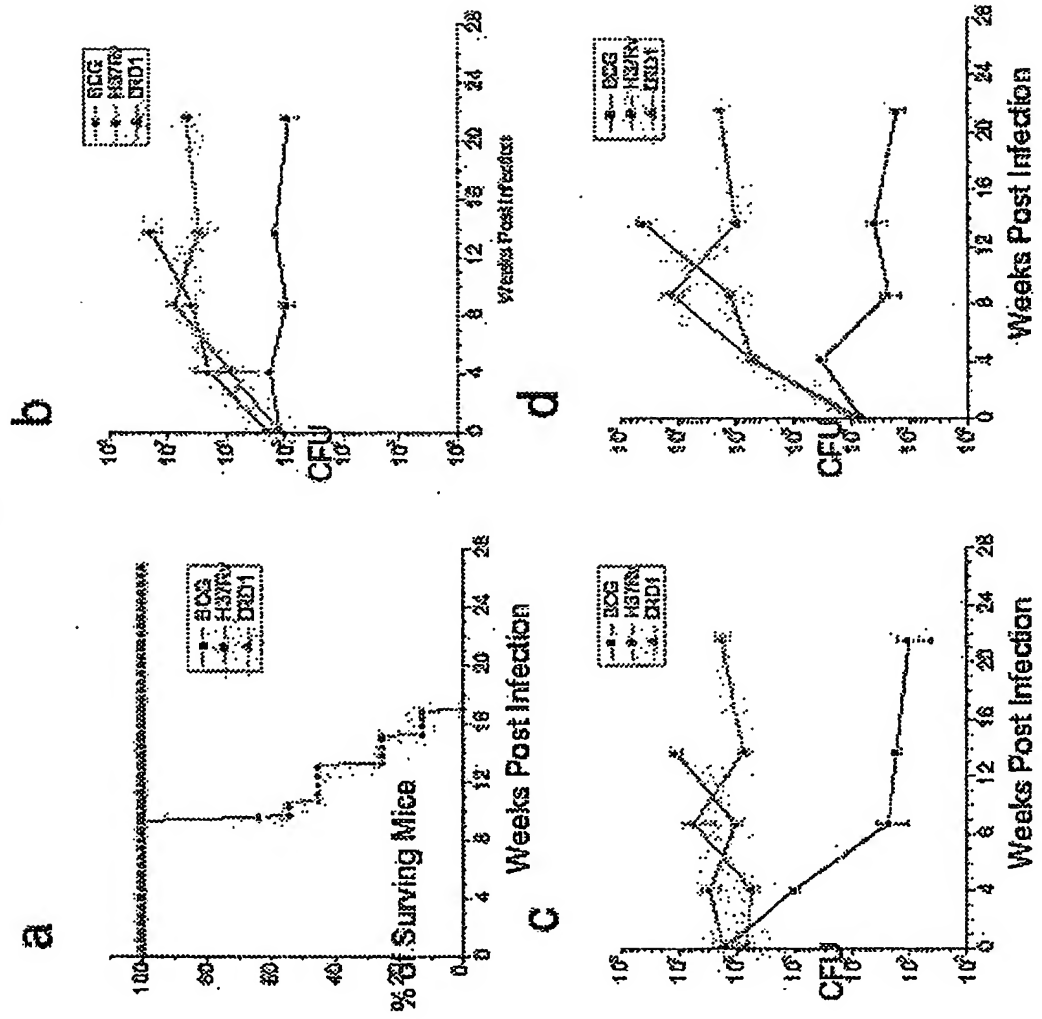


Fig. 5

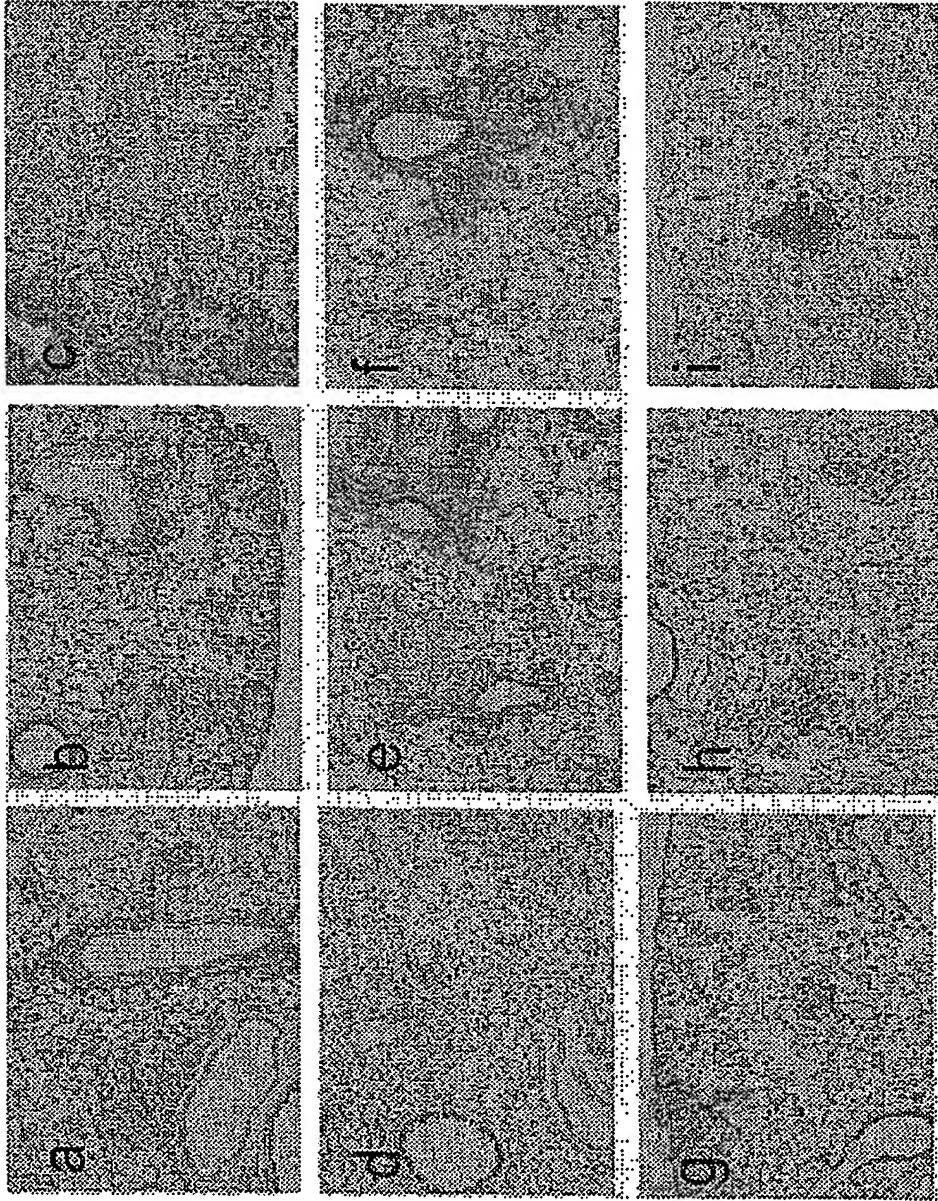


Fig. 6

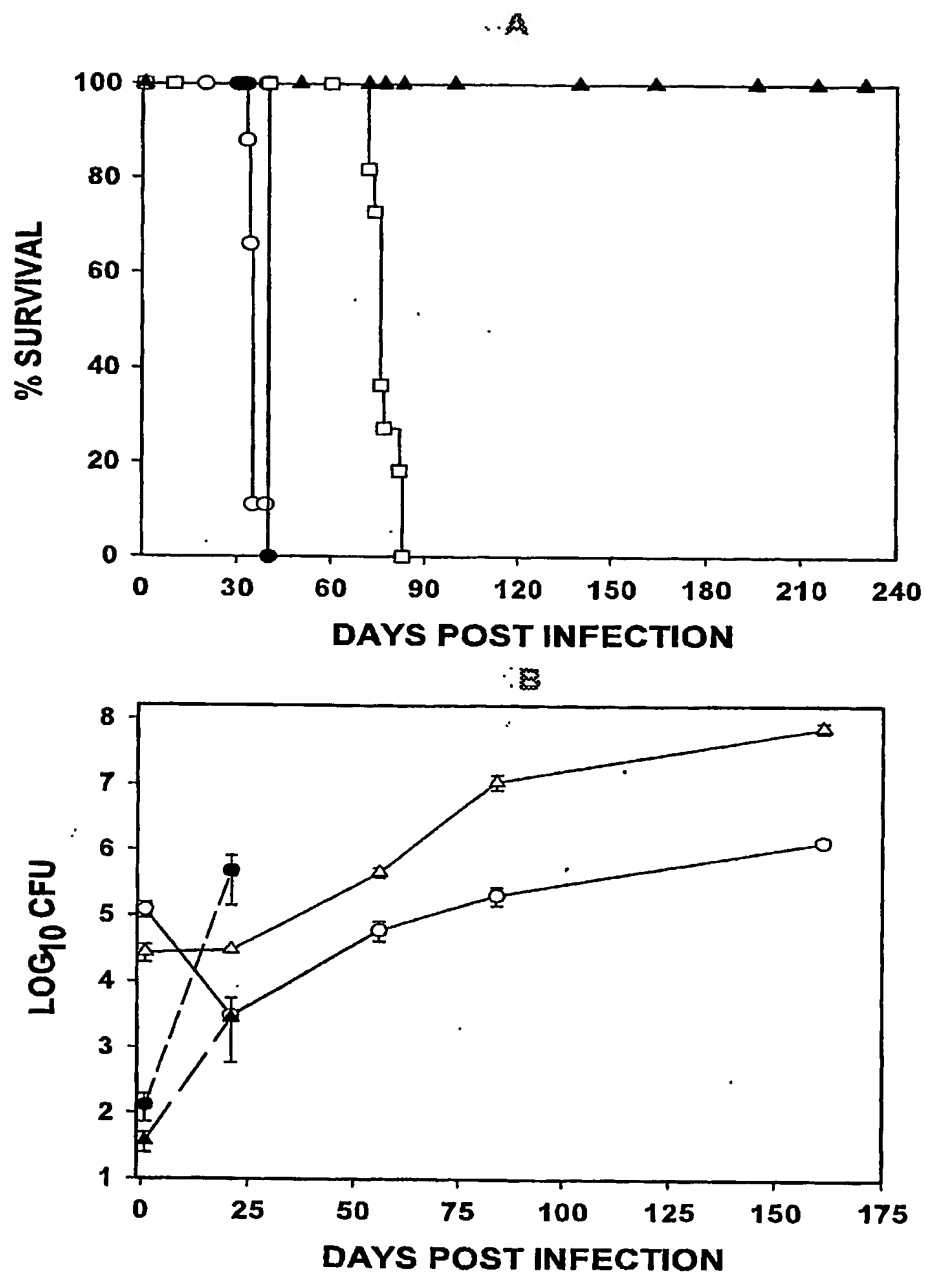


Fig. 7

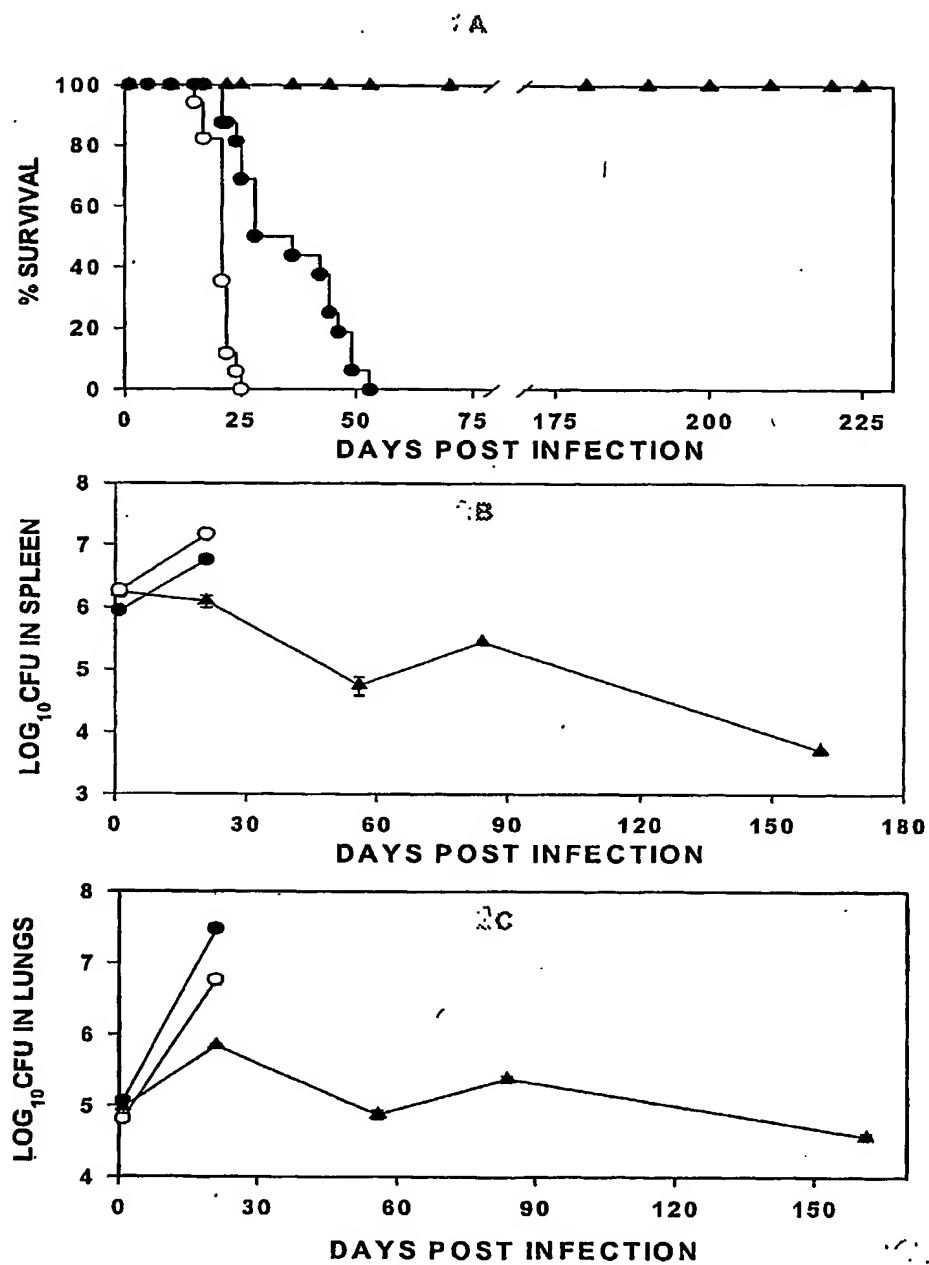


Fig. 8

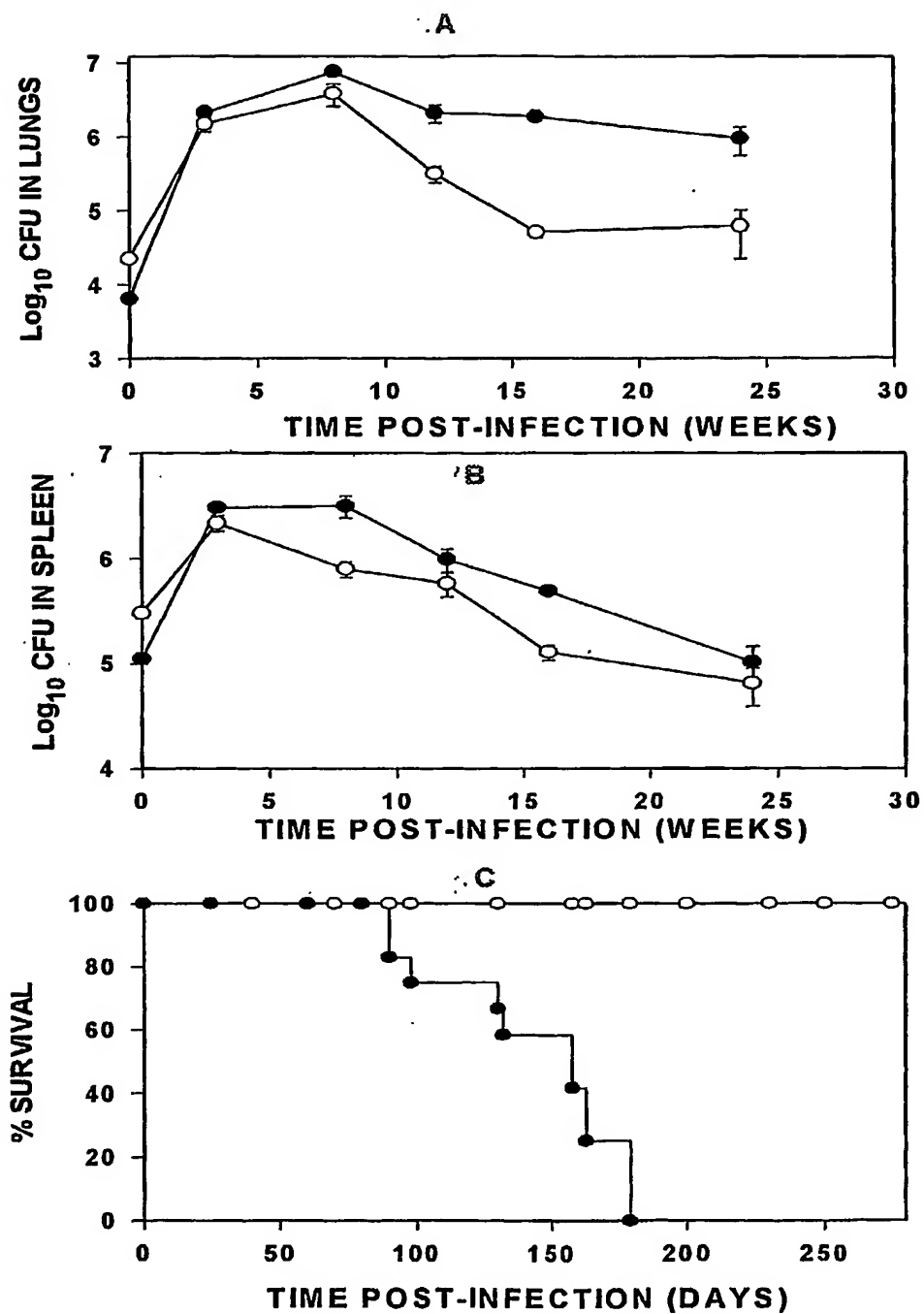


Fig. 9

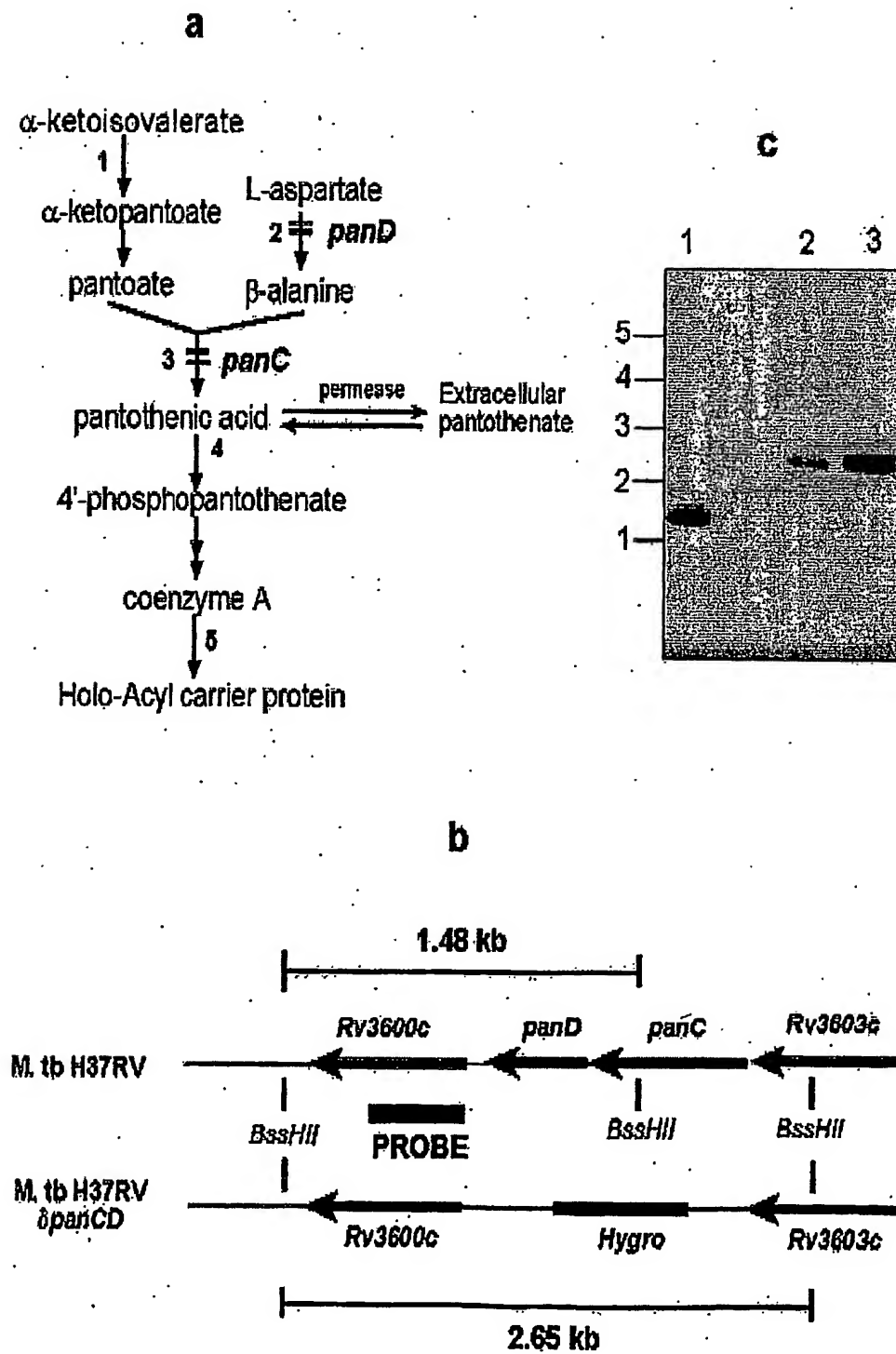


Fig. 10

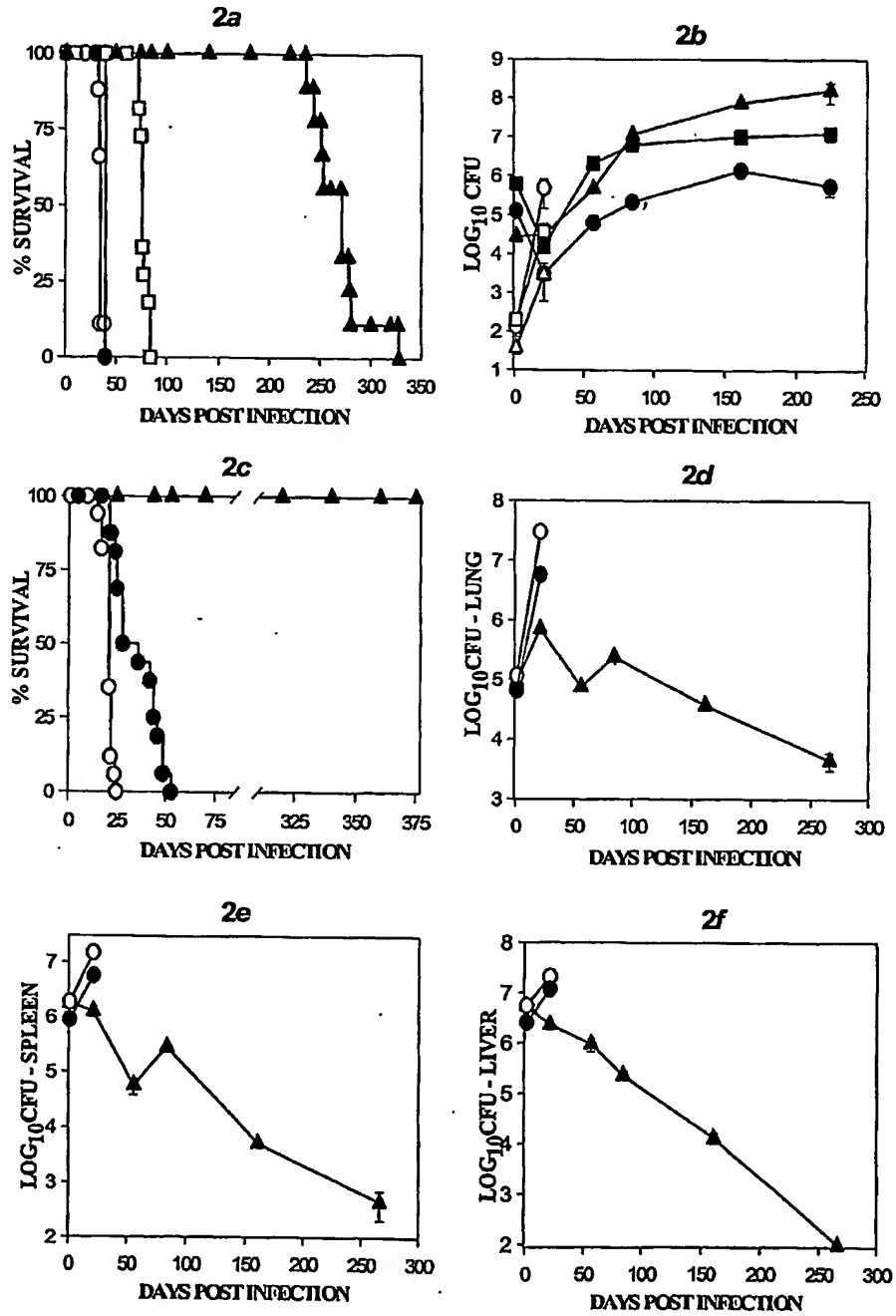


Fig. 11

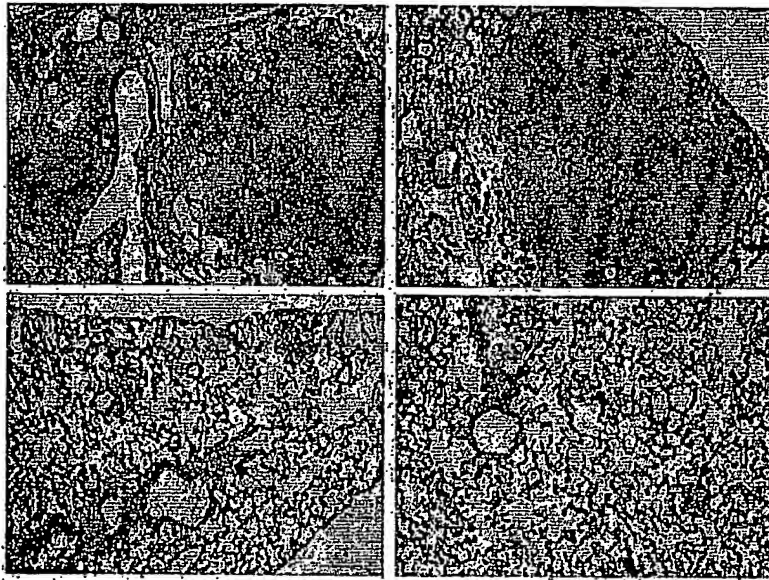


Fig. 11

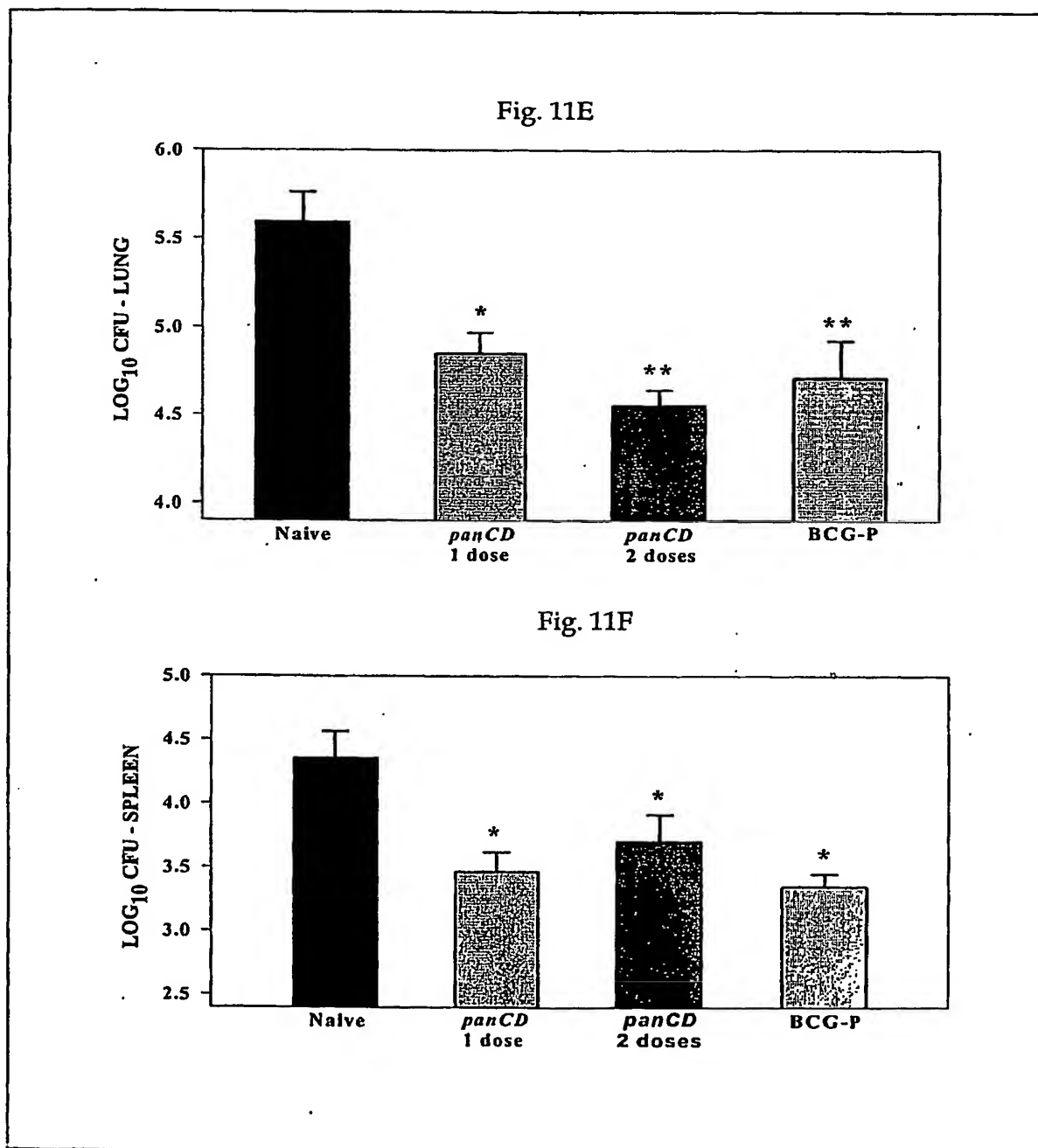


Fig. 12



Fig. 13

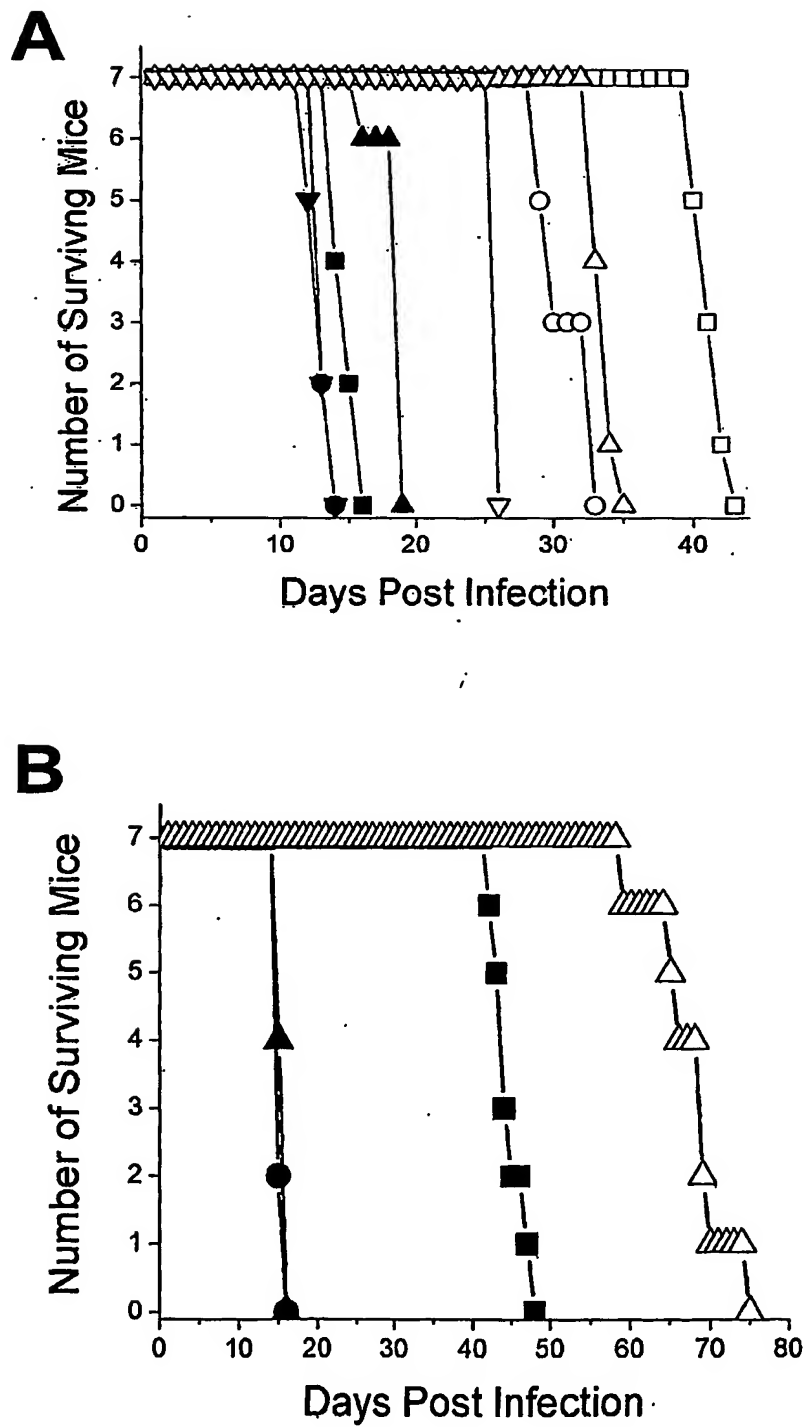


Fig. 13C

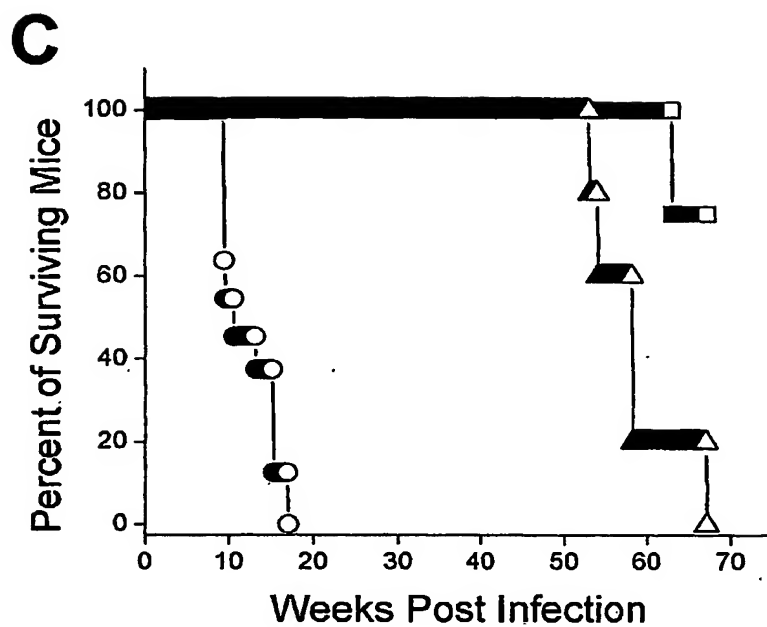


Fig. 14

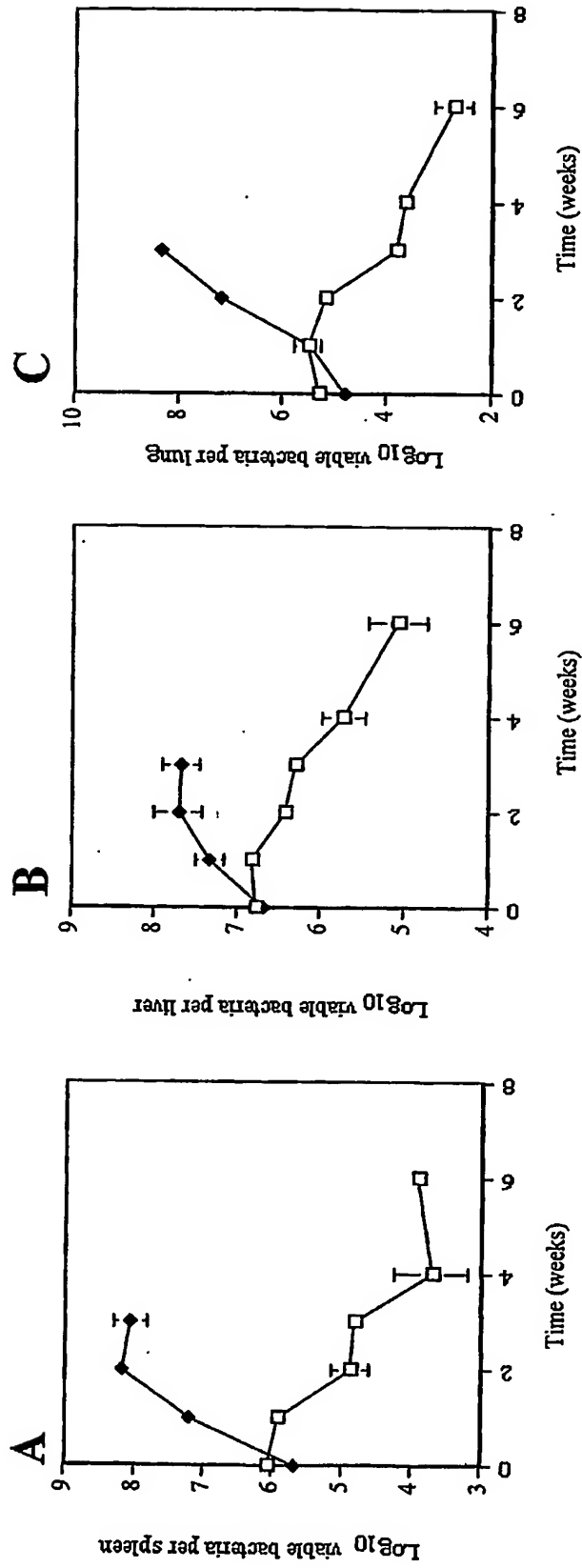


Fig. 15

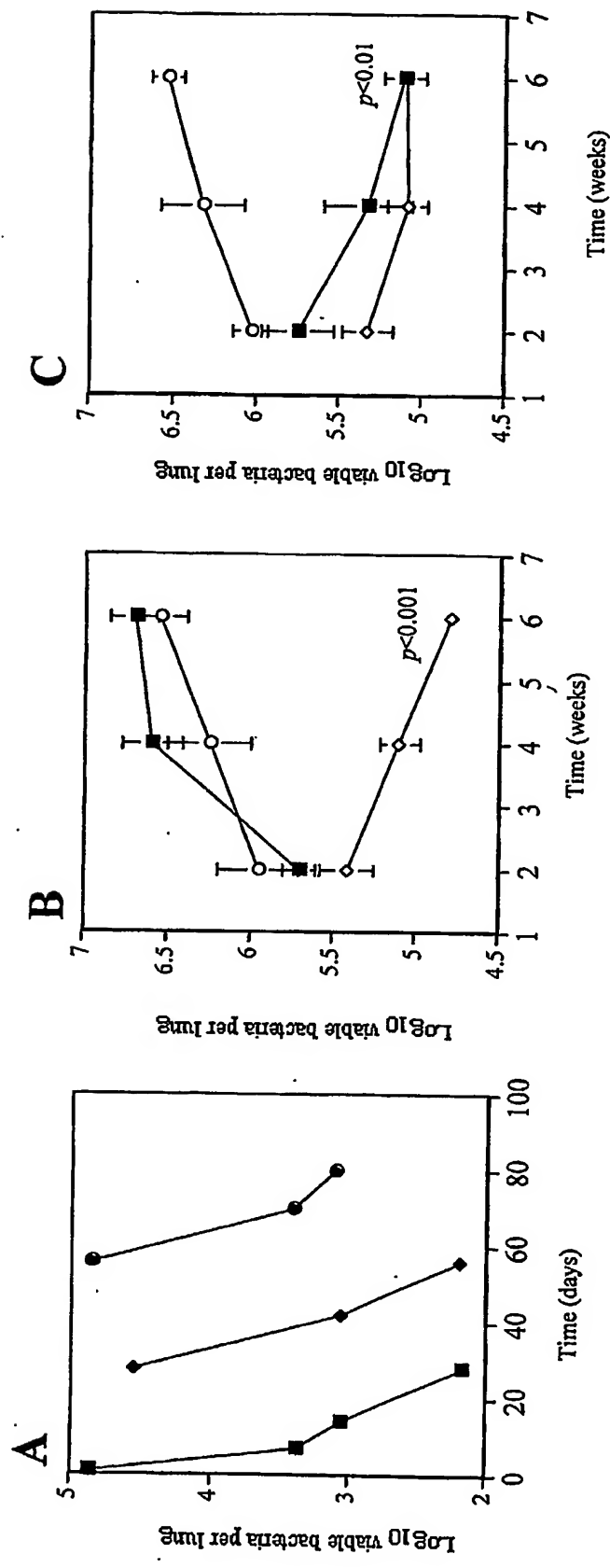


Fig. 16

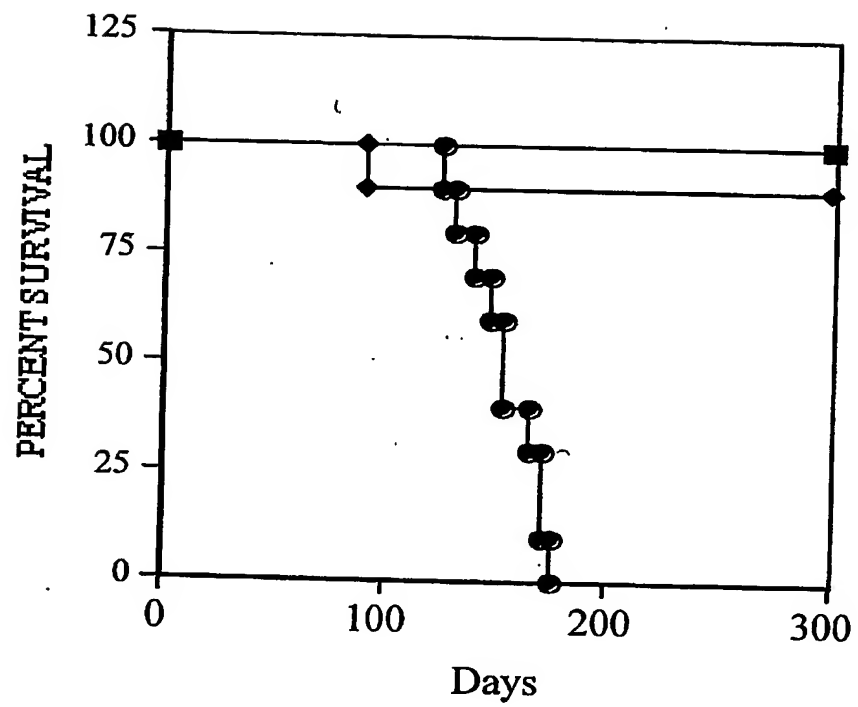


Fig. 17

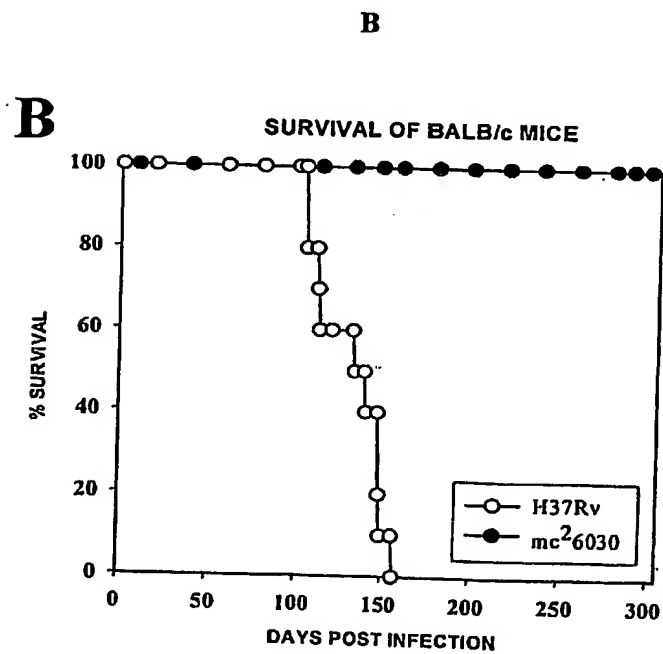
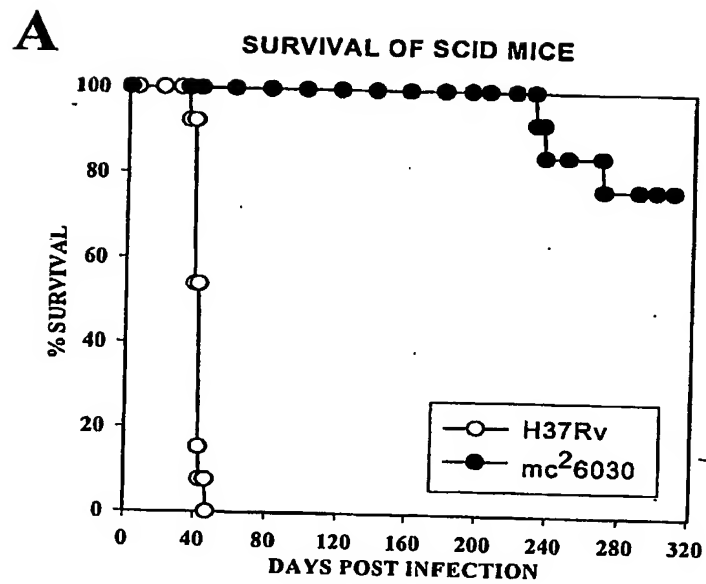


Fig. 18

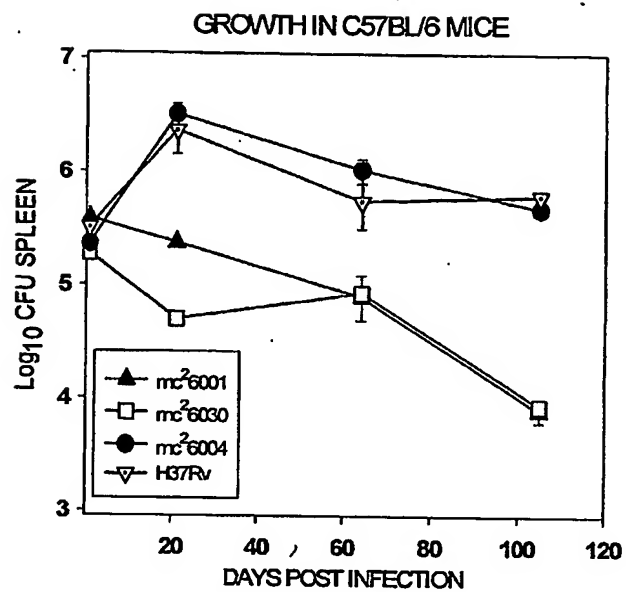
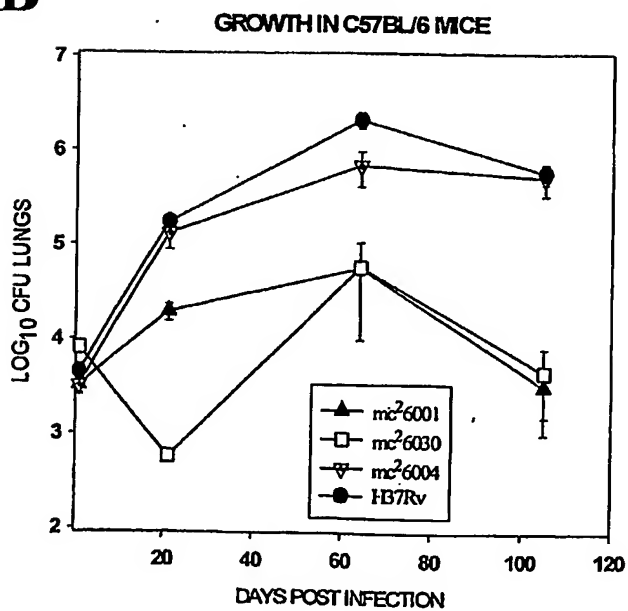
A**B**

Fig. 19

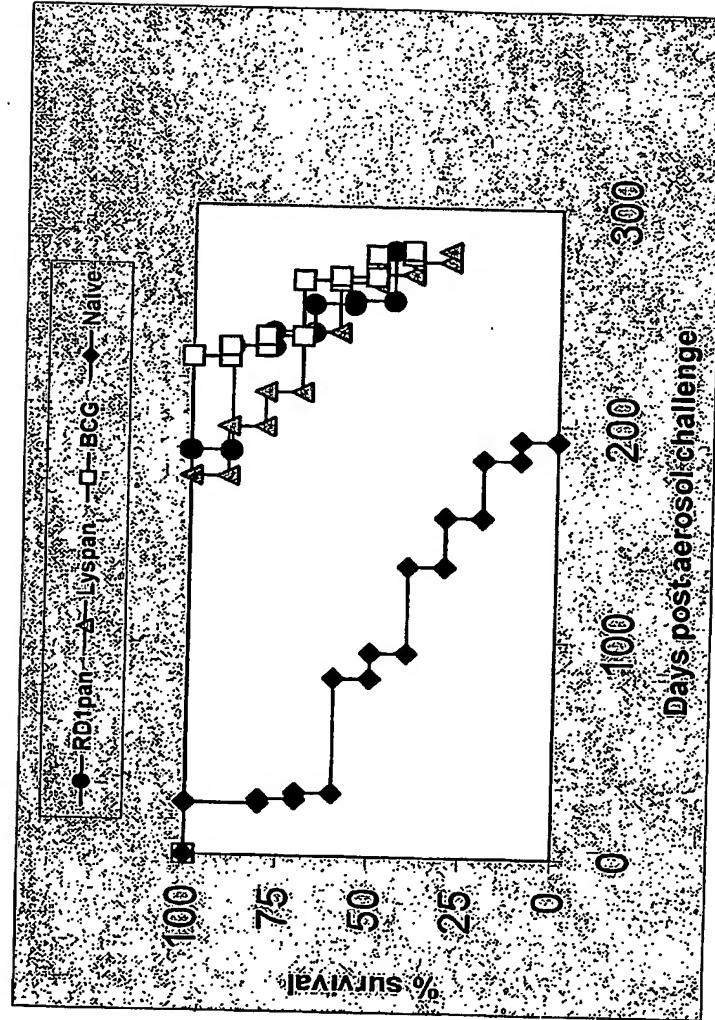


Fig. 20

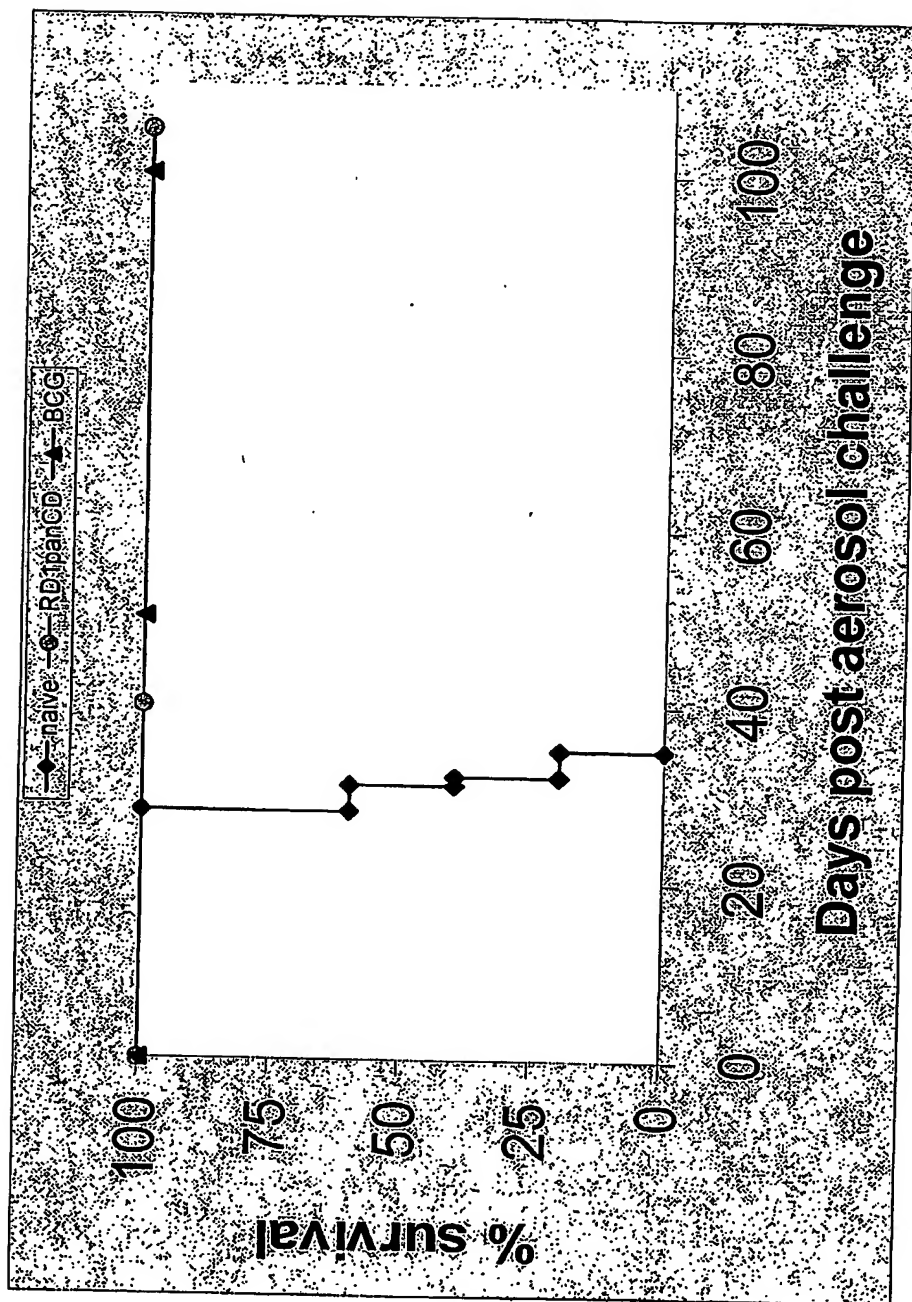


Fig. 21

